A THEORETICAL ANALYSIS OF RADIO-STRONTIUM METABOLISM AND DEPOSITION IN HUMANS

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A THEORETICAL ANALYSIS OF RADIO-STRONTIUM METABOLISM AND DEPOSITION IN HUMANS

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Summary

With available data on the calcium (Ca) content of the human body and present knowledge of Ca and ^{90}strontium (^{90}Sr) physiology as a basis the O R (body/diet) has been calculated from birth to adult age Different sets of values for O R (body/diet) have been obtained by varying the values for Ca intake or digestive juice Ca (i e by varying the true Ca absorption) and by using different values for O R (absorption) The formulas used and an example of the details in the calculations have been given allowing for corrections should other values prove to be more correct

Assuming a constant ingestion of ^{90}Sr in proportion to Ca from the time of birth to adult age a maximal value for O R (body/diet) is reached at one year of age Next this ratio declines by about 20 to 50 per cent in the course of the following ten to twenty years

The O R (body/diet) may be as high as 0 6 on low Ca diets Such a value may also be reached in some children living on high Ca-intakes equal to the recommended allowances in U S A The reason is that O R (absorption) may be higher than the usual average of 0 5 which results in a final O R (body/diet) of 0 25

When ^{90}Sr ingestion starts in children at increasing age the final O R (body/diet) is reduced The values calculated in children below the age of ten years are considerably higher than the values calculated in the older children, the values for O R (body/diet) decline fairly sharply after the age of ten years under such circumstances

The values for the concentration of ^{90}Sr in the diet of the eastern United States for the nine year period 1953 to 1961 were used in one set of calculations This resulted in a set of values for ^{90}Sr concentrations in human bones from the age of one to twenty years which agree fairly well with the values obtained by direct analysis of the ^{90}Sr content in human bone samples collected from the same region

Contents

Introduction

The nuclear test series conducted over the last years have increased the surface burden of radioactive fission products in the Northern Hemisphere Among the fall out isotopes ^{90}strontium is of importance because of the possible biological hazard caused by the retention of this element in the skeleton

The concentration of ^{90}Sr (expressed in μμc ^{90}Sr per g Ca) has been examined in human bones over a number of years (KULP, ECKELMANN and SCHULERT 1957, ECKELMANN, KULP and SCHULERT 1958 KULP SCHULERT and HODGES 1959 1960 KULP and SCHULERT 1962) The highest concentrations are found in the very young children and the concentrations of ^{90}Sr in children and teenagers are higher than in adults living in the same area An increase in the concentrations of ^{90}Sr has been observed in human bones taken from all age groups over the years between 1955 and 1960 An increase was also observed in the dietary concentrations of ^{90}Sr up to 1958–59 but it decreased in the years 1959–61 A new increase in the dietary concentrations of ^{90}Sr (Agricultural Research Council 1963) and in the concentrations of ^{90}Sr in human bones (Medical Research Council 1963) has been observed over the last year as a result of the tests in the autumn 1961 and 1962

Except perhaps in the very young children the concentration of ^{90}Sr in the human skeleton has not reached the maximal value (sometimes termed steady state or equilibrium) The time required in the different age groups for the establishment of a maximal ^{90}Sr/Ca ratio in their skeletons provided the dietary ratio of ^{90}Sr/Ca remains constant over a period of years is not precisely known The *radiation dose received by the body from ^{90}Sr in the skeleton* depends upon the concentration of ^{90}Sr in the bones and the time of exposure It is of great importance in the evaluation of the harm ful effects caused by the skeletal retention of ^{90}Sr to be able to predict the future body burden under a given set of circumstances

We have attempted to construct theoretical models for the cumulative retention of ^{90}Sr in children and young persons from 0–20 years of age calculated under different sets of conditions These models will assist in the evaluation of the possible hazards caused by the presence of ^{90}Sr in human bones and are presented in the following

THE ESSENTIALS OF CALCIUM PHYSIOLOGY

A brief review may be useful for readers not familiar with the field

At birth the skeleton contains about 30 g calcium From birth to adult age man accumulates 1000 to 1400 g Ca in the body (NICOLAYSEN EEG-LARSEN and MALM 1953) This range applies to nations in which the average adult body weight of the reference man is 70 kg In members of races with smaller body build the total calcium is expected to be proportionally lower

The average daily accumulation from birth to adult age (a period of 20 years is used) is 170 to 200 mg Ca Periods of rapid growth are associated with higher retention and vice versa Adaptation will compensate with a more efficient absorption when for a time retention has not been in proportion to the growth of the skeleton In adults balance is maintained when sufficient calcium is consumed with the food The minimum requirement for maintaining balance in adults has not been satisfactorily established

Useful data on balance in adult men at various levels of intake are found in the long term study of MALM (1958) When the daily intake was 900 mg and the men were in balance the urinary Ca varied between 100 and 400 mg Some men excrete over years about 100 mg others about 400 mg Those who excrete 100 mg Ca in the urine absorb (net) 100 mg and those who excrete 400 mg Ca absorb (net) 400 mg When the Ca intake was reduced by 50 per cent the average urinary Ca output was reduced by about 30 mg daily

In children the values for urinary calcium can be derived from KNAPP'S data (1947) At one year of age the mean is about 35 mg daily and twice the standard deviation is about 80 mg At five years of age the corresponding figures are 60 and 150 mg

These figures are representative when approximately the recommended daily allowances of calcium are consumed On much lower Ca intakes the figures will be lower

THE ABSORPTION OF CALCIUM

On very low intakes of Ca at an early age the absorption can be nearly complete However, it is generally incomplete and it is subjected to a dual regulation by vitamin D and the endogenous factor The reader is referred to NICOLAYSEN *et al* (1953) and MALM NICOLAYSEN and SKJELKVALE (1955) for additional information on the effect and interaction of these two factors Here it must suffice to state that vitamin D is the primary regulator of calcium absorption In the vitamin D free state the absorption is reduced to very low rates, and net absorption may be negative Adaptation with the aid of the endogenous factor cannot develop At low levels of cal cium in the diet adaptation with the aid of the endogenous factor will occur fairly rapidly when vitamin D is present in sufficient quantity in the body and the efficiency of absorption increases

The terms *absorption* and *net absorption* have been used above to designate calcium intake minus calcium excreted in the faeces *True absorption* is defined as follows

Calcium is secreted with all digestive juices into the gastro-intestinal tract and mixed with calcium consumed with the food From this mixture a fraction is absorbed

$$Ca_{intake} + Ca_{secreted} - X(Ca_{intake} + Ca_{secreted}) = Ca_{faeces}$$

ENDOGENOUS CALCIUM

The fraction of calcium which is excreted with the faeces is a mixture of calcium of immediate exogenous origin i e non-absorbed food calcium and of non absorbed digestive juices calcium The term endogenous was introduced by NICOLAYSEN (1937) to indi cate the amount of body calcium lost with the faeces Obviously no calcium is inborn and to introduce the term endogenous to include urinary calcium serves no useful purpose

According to the above (the factors regulating the speed of absorption and the equation) the endogenous calcium in the faeces will vary with a number of variables for example the amount of calcium eaten (a simple dilution phenomenon), the status of physical activity (a lumberjack who consumes 6000 calories will probably secrete at least twice more digestive juices than a sedentary worker, and consequently the $Ca_{secreted}$ will probably be at least doubled), etc

Table 1 is instructive with regard to dilution

Table 1

CALCULATED ENDOGENOUS CALCIUM IN FAECES IN MAN AT VARIOUS LEVELS OF CALCIUM INTAKE AND WITH DIGESTIVE JUICES CALCIUM ASSUMED TO BE 300 600 OR 900 MG DAILY (SUBJECTS NOS 790 AND 502 FROM MALM S (1958) DATA)

Days	Intake	Faeces	Balance	Digestive juices Ca 300	600	900
				Endogenous Ca		
112	1416	1139	26	198	336	440
140	910	662	− 8	164	263	330
238	443	277	−18	110	160	184
126	369	205	− 5	92	131	146
226	942	605	100	146	234	296
220	436	323	−88	132	186	218
206	454	252	− 7	96	132	153

The values are in mg Ca per day

Clearly the true absorption is the one important variable to find in any actual experiment An accurate estimate of the $Ca_{secreted}$ is then required When a figure for $Ca_{secreted}$ is inserted in the equation the endogenous Ca in the faeces can readily be calculated

The practice of extrapolation from linear regression of retention on intake estimated at two or more levels of intake to find endogenous loss at zero intake is beset with errors The term endogenous Ca in the faeces served a useful purpose 25 years ago It is not an important parameter in calcium physiology

DIGESTIVE JUICES CALCIUM

The reader is referred to MALM (1958) and to GRAN (1960) for more comprehensive information GRAN (1960) using ^{45}Ca performed direct experiments on rats He found 1) a 250 per cent variability in the digestive juices Ca at the same level of food intake 2) the digestive juices calcium was doubled when the food intake was doubled 3) digestive juices calcium was linearly related to plasma ionized calcium concentration

In man a few direct estimates have been made with the use of radiocalcium Values up to 1 9 g Ca per day have been reported (FINE and LASZLO 1957) whereas BLAU *et al* (1957) found values of

0 14 and 0 36 g On the other hand, numerous observations in man indicate that high values may be a fairly frequent occurrence

No 12 in MALM S (1958) group of men lost at least 500 mg digestive juices calcium daily in the faeces over a period of 28 days In two gastrectomized patients (NICOLAYSEN and RAGAARD 1955) the net absorption was minus 375 and 440 mg calcium daily in periods of 17 days Thus they must have secreted at least the same number of mg Ca with the digestive juices in spite of a very moderate food intake

Clearly much work remains to be done in this field with the aid of the methods which permit direct estimate of digestive juices calcium

In the theoretical analysis of the ^{90}Sr problem we have used chosen values for digestive juices calcium as will appear from the following

CALCIUM INTAKE

Calcium intake varies greatly in different countries (F AO 1960) The daily calcium intake in the Far East may be as low as 300–400 mg whereas the intake in U S A and Western Europe may well exceed the recommended allowances of the National Research Council (1958) Dietary surveys in Norway indicate that the calcium intake exceeds the recommended dietary allowances by approximately 200 mg daily Since the Ca intake in the different parts of the world may vary with a factor of three to four, it follows that the absorption from the intestine will vary from almost complete absorption to a fraction of the amount ingested

SOME ASPECTS OF STRONTIUM METABOLISM

The metabolism of strontium is intimately connected with the metabolism of calcium but the two elements are handled differently by the body in many of the metabolic processes This differential behaviour of strontium and calcium can be quantitatively expressed as the strontium calcium *Observed Ratio* (abbreviated O R)

$$\text{O R (sample/precursor)} = \frac{\text{Sr/Ca}_{\text{sample}}}{\text{Sr/Ca}_{\text{precursor}}}$$

We have chosen to express our results by the O R (body/diet), the results can easily be converted to strontium units (μμc ^{90}Sr per g Ca) when the dietary concentration of ^{90}Sr is known

For a detailed review of the metabolism of strontium reference may be made to the Reports of the United Nations Scientific Committee on the Effects of Atomic Radiation (1958 1962) The following aspects of strontium metabolism are important for the understanding of the calculations which are to be presented and the premises on which these calculations are based

The experiments of COMAR *et al* (1957) WASSERMAN *et al* (1958) and GRAN (1960) demonstrate that a constant level of Sr/Ca is rapidly established in the blood plasma of man, goat and rats following the daily ingestion of radio strontium in constant proportion to calcium Strontium is removed from the blood stream by deposition (accretion) of bone mineral ionic exchange reactions with the skeleton urinary excretion and secretion with the digestive juices The resorption of bone salt by skeletal remodelling will tend to reduce the Sr/Ca ratio in the plasma as long as the Sr/Ca ratio is lower in the resorbed bone compared with the ratio in the plasma It follows that the O R (plasma/diet) is lower than the O R (absorption)

The O R (bone/diet) in the newly accreted bone must necessarily be equal to the O R (plasma/diet) since no discrimination apparently takes place in the transfer of Sr and Ca from the blood plasma to the newly formed bone The O R (plasma/diet) is not well defined in humans this ratio can be estimated approximately in the following way

a) O R (plasma/diet) is equal to the O R (body/diet) when strontium is evenly distributed in the skeleton This has been shown to be the case in actual experiments in rats (GRAN 1960)

b) Determinations of the O R (body/diet) for stable strontium have given a value of 0 25 in Great Britain (BRYANT *et al* 1958), more limited materials from U S A and Canada have given values *from 0 25 to 0 5* and in Japan a *ratio of 0 65 has been reported* (FAO 1960)

c) Experiments in man and animals have given values for the O R (body/diet) in the range from 0 18 to 0 35 in most cases most of the reported values being grouped around 0 25

There is thus good evidence to support the use of a value equal to 0 25 for the O R (plasma/diet) In the calculations presented below

we have used this value except when the O R (body/diet) derived by calculation for the preceding year was higher than 0 25, under these circumstances O R (plasma/diet) was given a value equal to the O R (body/diet) calculated for the preceding year

The blood plasma will rapidly establish an equilibrium with the exchangeable part of the skeleton After an initial phase of a few days, the skeleton will retain only negligible amounts of strontium by exchange reactions provided the plasma ratio of Sr/Ca remains constant

Relatively more strontium than calcium is excreted in the urine because of the discrimination against strontium in the tubular reabsorption The O R (urine/plasma) seems to vary considerably in different species, this ratio will also vary with age etc In the calculations we have used a value of 1 0 for the O R (urine/diet) i e a value of 4 for O R (urine/plasma) when O R (plasma/diet) is 0 25

Strontium is also eliminated from the body by the faecal route even after the ingestion of strontium has been discontinued. This route of elimination is by no means insignificant, quantitatively it may be more important than the urinary excretion depending upon the experimental conditions The reason is that Sr is secreted together with Ca in the digestive juices In the absorption of Ca from the intestine the absorptive discrimination against strontium results in a preferential reabsorption of Ca Consequently the state of Ca absorption is of importance for the endogenous excretion of Sr in faeces In any case relatively more endogenous Sr than endogenous Ca will be found in faeces It is not known if the secretory glands of the digestive tract discriminate against or preferentially eliminate Sr We have assumed that the secretory glands do not discriminate in any such way i e that the Sr/Ca ratio in the digestive juices is identical to the Sr/Ca ratio in the blood plasma

CALCULATIONS

Calcium in faeces is a mixture of non absorbed food calcium and not reabsorbed digestive juices calcium

$$Ca_{faeces} = Ca_{intake} + Ca_{secreted} - X(Ca_{intake} + Ca_{secreted}) \quad (1)$$

faecal calcium and λ is the absorption coefficient for calcium (see NICOLAYSEN *et al.* 1953)

The net amount of calcium absorbed is calcium eaten minus calcium in faeces the net absorption is numerically equal to the sum of calcium retained and the urinary loss of Ca (Ca_{urine}) hence it follows

$$Ca_{intake} - Ca_{faeces} = Ca_{retained} - Ca_{urine} \qquad (2)$$

We can eliminate the term for Ca_{faeces} by combining equations (1) and (2) which will give the following expression for λ

$$\lambda = \frac{Ca_{retained} + Ca_{secreted} - Ca_{urine}}{Ca_{intake} + Ca_{secreted}} \qquad (3)$$

It follows that the true absorption of calcium is

$$Ca_{absorbed} = \lambda\,(Ca_{intake} + Ca_{secreted}) \qquad (4)$$

since the true calcium absorption equals the denominator in eq (3)

The true absorption of ⁹⁰Sr can be calculated in the same way as described for calcium however since the absorption of strontium is related to that of calcium the absorption coefficient for strontium is

λ O R. (absorption)

where λ is the absorption coefficient for calcium and O R. (absorption) indicates the discrimination against strontium in the process of absorption in the intestine. The true absorption of strontium is given by the following expression

$$^{90}Sr_{absorbed} = (^{90}Sr_{intake} - {}^{90}Sr_{secreted})\ \lambda\ O\ R.(absorption) \qquad (5)$$

The symbols are the same as for calcium. The result is expressed in strontium units (μμc per g Ca)

The amount of ⁹⁰Sr in the different terms in equation (5) can readily be calculated from the equivalent terms for calcium multiplied by the corresponding concentrations of ⁹⁰Sr expressed in units per g Ca. For example the total amount of ⁹⁰Sr secreted with the digestive juices is calculated as follows

$$^{90}Sr_{secreted} = Ca_{secreted}\ \ O\ R.(plasma\ diet)\ \ C \qquad (6)$$

where C is the concentration of strontium per g Ca in the diet (μμc per g Ca).

The total amount of strontium that is retained in the body during a given period can be calculated from

$$^{90}Sr_{increment} = {}^{90}Sr_{absorbed} - {}^{90}Sr_{secreted} - {}^{90}Sr_{urine}, \quad (7)$$

where the urinary excretion of strontium can be calculated from

$$^{90}Sr_{urine} = Ca_{urine} \cdot O.R.\ (urine/diet) \cdot C \quad (8)$$

The O R (body/diet) can next be calculated

$$O.R.\ (body/diet) = \frac{^{90}Sr_{body}}{\text{total body Ca}}$$

where $^{90}Sr_{body}$ is the total amount of ^{90}Sr in the body In the actual calculations the yearly increment of ^{90}Sr was calculated according to the equations given above (5-8) and the O R (body/diet) was next calculated for each year

In our calculations we have assumed that the following conditions are fulfilled

a) Complete mixing of calcium and strontium from the different dietary sources

b) Complete mixing of ingested calcium and strontium with calcium and strontium secreted with the digestive juices

c) Calcium and strontium are handled identically in the secretion of digestive juices i e no discrimination or preferential elimination of strontium takes place in the secretory glands of the digestive tract

d) The O R (absorption) and O R (urine/diet) remain constant in the first 20 years of life i e these discriminations are independent of age

We have not taken into account the fact that the human infant is contaminated with ^{90}Sr at the time of birth and that radioactive decay will reduce the body burden of ^{90}Sr

We have used the values of LEITCH and AITKENS (1959) for body calcium and calcium increment in young males and the National Research Council s (1958) recommended dietary Ca allowances representative for the calcium intake in the Western World The data for urinary calcium have been derived from KNAPP (1948) giving the variations in urinary calcium at different levels of Ca

Table 2

THE VALUES USED IN THE CALCULATIONS FOR BODY CALCIUM, CA INCREMENT, RECOMMENDED DIETARY ALLOWANCES, DIGESTIVE JUICES CA, URINARY CA ETC

Age Years	Body weight kg	Total body Ca g	Ca increment mg/day	Digestive juices Ca mg/day	Recommended Ca allowance mg/day	Urinary Ca at recommended Ca intake mg/day	True Ca absorption per cent	Assumed Ca intake in Norway mg/day
0	3.5	28.5						
1	9.4	84.3	153	400	800	35	49.0	800
2	12.4	116.4	88	400	1000	47	38.2	1000
3	14.6	141.6	69	400	1000	55	37.4	1000
4	16.7	167.0	70	500	1000	60	42.0	1100
5	19.0	196.3	80	500	1000	68	43.2	1100
6	21.2	225.6	80	500	1000	75	43.7	1200
7	23.9	263.4	104	500	1000	82	45.7	1200
8	26.5	301.6	105	600	1000	90	49.7	1200
9	29.4	346.6	123	600	1000	98	51.3	1300
10	32.6	399.0	144	600	1000	102	52.9	1300
11	36.2	461.2	170	600	1200	120	49.4	1400
12	40.2	534.7	201	700	1200	124	53.9	1400
13	45.4	637.0	280	700	1200	146	59.3	1500
14	51.0	755.3	324	700	1400	164	56.6	1500
15	57.0	892.1	375	700	1400	179	59.7	1500
16	62.6	1028.5	374	800	1400	189	62.0	1600
17	65.9	1113.7	233	800	1400	193	55.7	1600
18	68.2	1175.1	168	800	1400	206	53.4	1600
19	69.4	1207.6	89	800	1400	207	49.8	1600
20	70.0	1223.6	44	800	1400	210	47.9	1600

The values are for boys

intake Precise values for Ca secreted with the digestive juices are not available especially in the case of children The figures used are given in Table 2 deviations from these appear in the text

In the calculations of the curves to be presented in the following chapters, we have tried to correlate our results with the observations available for stable strontium and next we have calculated the uptake of radiostrontium in humans exposed to a contaminated diet over a few years

In the first instance we have assumed that the child eats a diet which provides strontium at a constant proportion to calcium in the first twenty years of life The curves calculated can serve as models for the O R (body/diet) in a population which has been continuously exposed to a constant dietary ratio of Sr/Ca throughout life

We shall discuss below the factors of primary importance for the calculations of a representative curve for the strontium retention in humans, i e the secretion of calcium and strontium with the digestive juices O R (absorption) the level of calcium intake etc

The ratio of ^{90}Sr to Ca varies considerably in the course of time Such variations are determined by a number of factors such as the amount of radiostrontium present in the atmosphere rate of fall out etc. The results of calculations of radiostrontium uptake in a population over a few years will be presented later (pp 30–34)

Factors affecting the retention of strontium in humans consuming a diet with $^{90}Sr/Ca$ constant in the first twenty years of life

THE SECRETION OF CALCIUM AND STRONTIUM WITH THE DIGESTIVE JUICES

The secretion of calcium and strontium with the digestive juices has been discussed on pages 12 and 15 It is essential to include these figures in the calculation of strontium retention

This is illustrated in Figure 1 The same value (0 5) was used for the O R (absorption) in the calculation of all three curves O R (urine/diet) was taken to be 1 0 Furthermore the calcium intake equalled the recommendations of the National Research Council (1958) and it was assumed that the dietary ratio of Sr/Ca remained constant. The following values were used for the digestive juices calcium

1) High values given in Table 2.
2) A value reduced to fifty per cent of 1)
3) The digestive juices calcium and strontium were set at zero an obvious error

The results appear from Figure 1 The use of $Ca_{secreted}$ in a two to one proportion results in nearly identical curves whereas neglecting $Ca_{secreted}$ in the calculation results in an O R. (body/diet) about 50 per cent smaller Table 3 is useful because other values for digestive juices Ca and Ca intake can also be introduced and other O R. (body diet) can readily be calculated

The explanation of the very close identity of curves a and b is as follows the true absorption of calcium is higher when high values are used for the digestive juices calcium however the amount of strontium secreted with the digestive juices will also be higher The

net result is that the retention of strontium will be almost equal in the two cases

This will be understood from calculations carried out according to the equations used The results for the first six years of life assuming high or medium values for digestive juice calcium, are given in Table 3 The figures for calcium retention and total body calcium are found in Table 2 Thus considerable variability in digestive juice calcium may occur without any appreciable effect on the O R (body/diet)

We have assumed that the Sr/Ca ratio in the digestive juices equals the ratio in the blood plasma Results derived from experiments in rats indicate that no major changes occur in the Sr/Ca ratio when calcium and strontium are secreted with the digestive juices (GRAN 1960)

The negative slopes of curves a and b in Figure 1 require a few comments A maximal value for O R (body/diet) was reached at the age of one year in both O R (body/diet) was next reduced to approximately 0 28 at the age of 10 years From the age of about fifteen years a second negative slope is found This finding is in full agreement with the experimental results in rats living on a diet with

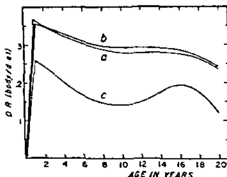


Fig 1 The O R (body/diet) as a function of variability in the secretion of calcium with the digestive juices

Curve a High values for digestive juices Ca see Table 2
Curve b Digestive juices Ca reduced to 50 per cent of a.
Curve c Digestive juices Ca taken to be zero

It is assumed that ^{90}Sr has been ingested at a constant proportion to Ca in the first twenty years of life The values used for total body Ca Ca increment Ca intake digestive juices Ca and urinary Ca are taken from Table 2 O R (absorption) is 0 5 O R (urine/diet) 1 0 O R (plasma/diet) 0 25 for the first year of life and thereafter given a value equal to O R (body/diet) derived by calculation for the preceding year.

Table 3

EXAMPLES ON THE CALCULATION OF O R (BODY/DIET)

	Age years	Calcium mg/day Intake	Urine	Digestive juices	True absorption	Strontium units[1]/day Intake	Digestive juices	Urine	True absorption	Retained	Increment units/year	Total ^{90}Sr in body units	O R (body/diet)
Figure 1 curve a	1	800	35	400	588	800	100	35	221	86	31390	31390	0 37
	2	1000	47	400	535	1000	148	47	219	24	8760	40150	0 34
High digestive	3	1000	55	400	524	1000	136	55	212	21	7665	47815	0 34
juices calcium	4	1000	60	500	630	1000	170	60	246	16	5840	53655	0 32
	5	1000	68	500	648	1000	160	68	251	23	8395	62050	0 32
	6	1000	75	500	655	1000	160	75	253	18	6570	68620	0 30
Figure 1 curve b	1	800	35	200	388	800	50	35	165	80	29200	29200	0 35
	2	1000	47	200	335	1000	70	47	149	32	11680	40880	0 35
Low digestive	3	1000	55	200	324	1000	70	55	144	19	6935	47815	0 34
juices calcium	4	1000	60	250	380	1000	85	60	165	20	7300	55115	0 33
	5	1000	68	250	398	1000	83	68	172	21	7665	62780	0 32
	6	1000	75	250	405	1000	80	75	175	20	7300	70080	0 31

[1] In arbitrary units

The corresponding values for total body calcium are found in Table 2

constant ^{90}Sr/Ca over a considerable period of time (GRAN 1960) The explanation is as follows the secretion of strontium with the digestive juices results in a reduction in the body Sr/Ca ratio since the body discriminates against strontium in the reabsorption from the intestine Additionally the kidneys eliminate strontium in preference to calcium Although the concentration of strontium per unit calcium is reduced in the body the total body content of strontium increases throughout the period of growth

The negative slopes in curves a and b in Figure 1 may be somewhat exaggerated It has been assumed that the skeleton and the blood plasma are in equilibrium and that strontium is evenly distributed in the skeleton The negative slope of the curve between one and ten years of age probably represents a biological reality since the skeletal turnover is very rapid in young individuals It must be expected that in the end the curves will be horizontal because the rate of skeletal turnover decreases with age The slopes found after the age of fifteen years (see Figure 1) may therefore not represent a biological reality

O R (ABSORPTION)

The intestinal discrimination against strontium varies considerably The most important factor which influences discrimination is the magnitude of true calcium absorption Almost complete absorption of dietary and secreted calcium results in very small amounts of calcium for dilution of radiostrontium The discrimination in such instances will be small on the contrary discrimination will be high when the true absorption of calcium is low

Double tracer experiments in rats with the aid of ^{45}Ca and ^{89}Sr are instructive (GRAN 1960) When 99 and 92 per cent of ^{45}Ca were absorbed the corresponding values for O R (absorption) were 0 88 and 0 77 respectively When only 36 per cent of ^{45}Ca was absorbed O R. (absorption) was reduced to 0 30

In experiments in man COMAR *et al* (1957) have observed O R (absorption) values as high as 0 78 SPENCER *et al* (1957) found an O R (absorption) of 0 46 and others have found values in the same range or lower

We have used three different values for O R (absorption) 0 3 0 5 and 0 7 respectively in calculations which resulted in the three

curves presented in Figure 2 It appears that the results for O R (body/diet) derived vary greatly with O R (absorption)

Sufficient evidence with the use of stable strontium has been produced to indicate that O R (absorption) will be between 0 25 and 0 5 The values for O R (body/diet) in curves a and b in Figure 2 correspond well to the O R (body/diet) derived from stable strontium observations Since stable strontium is ingested with the food from birth and onwards in a fairly constant proportion to calcium the results from such observations provide a valuable control of our method of calculation

Since the use of 0 5 for O R (absorption) resulted in a curve for O R. (body/diet) which corresponds to that derived from studies of stable strontium in the bodies of the British population (BRYANT *et al* 1958) and to experimentally determined ratios in a number of animals we felt it justified to use 0 5 as the value for O R. (absorption) in the calculations to follow However, the absorption of calcium varies between individuals and in a number of children it will be high at least periodically Under such circumstances the O R (body/diet) will probably correspond to the curve a in Figure 2

Considerable deviations from the calculated curves may therefore be expected to occur within a population and it is probable that a number of children will achieve an O R (body/diet) considerably above the 0 25 which is in common use among radiobiologists when they calculate the ^{90}Sr cont^nt of the body corresponding to a given fall out over a period of years

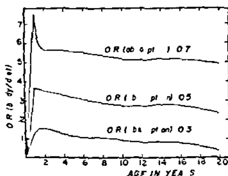


Fig 2. The O R (body/diet) as a function of variability in the O R (absorption) The O R. (absorption) was varied as indicated in the Figure otherwise the legend to Figure 1 also applies to this Figure

The data in Table 2 indicate that the true absorption of calcium is highest in the period of most rapid growth When the calcium intake is equal to the recommended dietary allowances of the National Research Council (1958) the true absorption of calcium varies between 38 2 per cent and 62 per cent in the first twenty years of life At lower levels of intake the true absorption increases

In long term experiments with rats (GRAN 1960) the O R (body/diet) varied with the true absorption of calcium and therefore with the dietary level of calcium WASSERMAN *et al* (1957) and PALMER *et al* (1958) also made such observations in rats

The daily calcium intake varies from less than 0 4 g in parts of Africa and Asia to more than 1 g in most of Europe and U S A Within different countries the daily intake of calcium varies between sections of the populations Fish and marine products are important sources of calcium in Japan cereals and vegetables in countries like India In other countries such as Norway and U S A milk and dairy products contribute more than 80 per cent of the total calcium intake (FAO 1960)

We have limited our calculations as will be seen below The O R (body/diet) was calculated for three levels of calcium in the diet with identical $^{90}Sr/Ca$ ratios

1) A high intake representing the average Norwegian intake (see Table 2) Figure 3 curve a

2) The recommended dietary allowances of calcium (National Research Council 1958) Figure 3 curve b

3) Fifty per cent of the intake in 2 above Figure 3 curve c

4) The effect of adding 0 5 g uncontaminated calcium to the daily intake used in 2 above Figure 3 curve d

It appears from Figure 3 that the calculated O R (body/diet) were nearly identical in the first two instances Curve a should probably be somewhat lower than curve b since O R (absorption) is reduced when the calcium intake increases However the difference in assumed daily calcium intake was small (see Table 2) and the curves are probably very nearly representative

Supplementation with 0 5 g of uncontaminated calcium depresses O R (body/diet) as expected Probably the correct curve should be

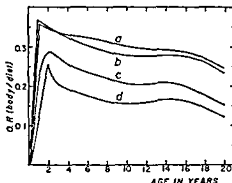


Fig 3 The O R (body/diet) as a function of variability in the calcium intake

Curve a High Ca intake
Curve b The U. S A recommended dietary allowance of Ca
Curve c Ca intake reduced to 50 per cent of b
Curve d The recommended dietary allowance (U S A) with daily addition of 0 5 g uncontaminated Ca

The legend to Figure 1 and the text should be consulted for further details O R. (absorption) is 0 5 in all cases

somewhat lower, since the O R (absorption) is reduced when the calcium level in the diet increases In consequence the calculated reduction of O R (body/diet) represents a minimal value for the reduction to be expected In the given calculation it was about 30 per cent possibly 40 per cent would represent a more correct value

Curve b in Figure 3 corresponds to curve b in Figure 1 The O R (body/diet) in this curve corresponds to the O R (body/diet) derived from studies of the retention of stable strontium in humans exposed to a relatively constant dietary Sr/Ca level throughout life

Curve c was derived with the aid of O R (absorption) of 0 5 However when the calcium intake is substantially reduced the O R. (absorption) increases and the O R (body/diet) derived is probably grossly below the true values

It is quite possible that the O R (absorption) under such circumstances may be as high as 0 7 or even somewhat higher Such calcium intakes occur only in populations living on low calcium diets However the body size of adult men is then much below the European standard man of 70 kg and the use of the figures in Table 2 introduces some errors Regrettably no precise information with regard to the Ca balance under such circumstances is available Ceylonese skeletons (for reference see NICOLAYSEN *et al* 1953) weighed about 20 per cent less than European skeletons, the errors

introduced may therefore be of moderate size Balance studies in such populations indicate extremely efficient absorption and low urinary Ca excretion (for reference see NICOLAYSEN *et al* 1953)

In spite of all uncertainties a calculation as given in Figure 4 and resulting in O R (body/diet) of 0 6 at the peak and falling slowly to about 0 5 may be fairly representative Other values can readily be introduced and other O R (body/diet) will result

Work with stable strontium in Japan indicates an O R (body/diet) of about 0 6 (FAO 1960) Probably our calculated O R (body/diet) at low levels of calcium in the diet of children is not grossly off the mark

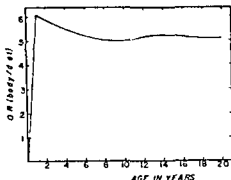


Fig 4 The O R (body/diet) as a function of a low Ca intake and reduced absorptive discrimination against strontium

It is assumed that ^{90}Sr has been ingested at a constant proportion to Ca in the first twenty years of life The values used for body Ca Ca increment and digestive juices Ca are taken from Table 2 The Ca intake is 50 per cent of the recommended allowance in Table 2 and the urinary excretion of Ca has been derived from KNAPP s data (1947) O R (absorption) is 0 75 O R (urine/diet) 1 0 and O R. (plasma/diet) 0 25 for the first year of life and thereafter given a value equal to O R. (body/diet) derived by calculation for the preceding year

THE DEPRESSION OF ^{90}SR ABSORPTION BY UNCONTAMINATED CALCIUM IN THE DIET OF ADULT MAN

In the preceding section the effect of adding uncontaminated calcium to the diets of children was calculated Frequently it has been suggested that the addition of uncontaminated calcium to bread would be a valuable aid in reducing ^{90}Sr in the body

We have made a calculation of the depressive effect to be expected in an adult man living on low intermediate or high calcium intake Three levels of uncontaminated calcium were used

Table 4

CALCULATED ABSORPTION OF ^{90}SR AT DIFFERENT LEVELS OF CA INTAKE

Food Ca mg/day	Ca supplement mg/day	Total Ca intake mg/day	Ca urine mg/day	True Ca absorption		^{90}Sr intake units	True ^{90}Sr absorption		Per cent depression of ^{90}Sr absorption
				mg/day	Per cent		units	Per cent	
500	0	500	185	985	75.8	500	190	38.0	
500	500	1000	200	1000	55.6	500	139	27.8	26.8
500	1000	1500	212	1012	44.0	500	110	22.0	42.1
500	1500	2000	234	1034	36.9	500	92	18.4	51.6
1000	0	1000	200	1000	55.6	1000	278	27.8	
1000	500	1500	212	1012	44.0	1000	220	22.0	20.9
1000	1000	2000	234	1034	36.9	1000	185	18.5	33.5
1000	1500	2500	250	1050	31.8	1000	159	15.9	42.8
1500	0	1500	212	1012	44.0	1500	330	22.0	
1500	500	2000	234	1034	36.9	1500	277	18.5	16.1
1500	1000	2500	250	1050	31.8	1500	239	15.9	27.6
1500	1500	3000	265	1065	28.0	1500	210	14.0	36.4

The calculations are for an adult man (70 kg) in Ca balance the digestive juices Ca is 500 mg/day It was assumed that the dietary concentration of ^{90}Sr was 1 arbitrary unit per mg Ca The dietary supplement is by uncontaminated Ca O R (absorption) is 0.5

The results appear in Table 4 As expected the effect is most marked when high amounts of calcium are added to the diet with a fairly low level of Ca Since the true absorption is very nearly the same in all instances, O R (absorption) is probably not reduced by the addition of even high amounts of calcium to the diet

When 1 g uncontaminated calcium has been added the depression achieved varies from 28 to 42 per cent Such an effect probably represents what can be maximally achieved in practice

The retention of strontium in humans consuming a diet with ^{90}Sr/Ca constant over a few years

In the preceding chapters we have considered the retention of strontium in children on the assumption that the dietary ratio of Sr/Ca was constant. The curves therefore correspond to the situation for stable strontium

After a nuclear explosion the ^{90}Sr in the diet increases to a maximum followed by a decrease if further tests are not carried out. We face the following situation children of varying age consume a ^{90}Sr-contaminated diet and their skeletons will retain radiostrontium until a maximal level of ^{90}Sr is established in the body In the following we have calculated these maximal levels for the different age groups following chronic exposure to a radioactive diet over several years and also the time required in order to establish these maximal ratios

In the first set of calculations we have assumed that the dietary ratio of ^{90}Sr/Ca is constant that the body does not contain ^{90}Sr at the beginning of radiostrontium ingestion and that the retained radiostrontium is evenly distributed in the skeleton The O R. (absorption) was taken to be 0 5 and a value of 1 0 was used for the O R. (urine/diet) The calcium intake equals the recommendations of the National Research Council (1958) The values given in Table 2 for total body Ca Ca retention urinary Ca and digestive juices Ca were used.

The results are presented in Figure 5 The curve for a new-born child has not been included curve b in Figure 1 should therefore be used for comparison. The maximal value reached for O R. (body/diet) is highest in the youngest children. The time required to reach the maximal O R. (body/diet) is shortest in the youngest children The negative slopes of the curves after the maxima are reached represent a biological reality observed in long term experiments with rats (GRAN 1960)

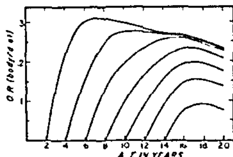


Fig 5 The calculated O R. (body/diet) in children who have started to eat a ^{90}Sr-contaminated diet at different ages

It is assumed that ^{90}Sr has been ingested at a constant proportion to calcium from the time of start on the ^{90}Sr-contaminated diet The values used for total body Ca Ca intake etc are found in Table 2 O R (absorption) is 0 5 O R. (urine/diet) 1 0 and O R (plasma/diet) is 0 25 or given a value equal to the O R (body/diet) calculated for the preceding year if this ratio is higher than 0 25

The ^{90}Sr/Ca in newly deposited (accreted) bone is identical to the ratio in blood plasma there being no discrimination against strontium in the transfer of the two elements from blood plasma into the bones The over-all skeletal ratio depends upon the total amount of Ca present in the skeleton including calcium in the non-radioactive bone structures Bone remodelling will in the course of time replace the uncontaminated structures with radiostrontium-containing bone The amount of calcium in the skeleton at the beginning of radiostrontium ingestion and the rate of skeletal remodelling are therefore of great importance for the validity of the calculated results The rate of skeletal remodelling decreases when growth declines and the calcium content of the skeleton increases with age until growth is discontinued

The calculated results for O R (body/diet) in children under the age of 10–12 years of age are most probably nearly correct The skeleton retains on the average less than 400 g during the first ten years of life and about 800 g in the next ten years In the children below 10–12 years the rate of skeletal remodelling is high and a relatively high proportion of skeleton is in equilibrium with the blood plasma by exchange processes

In the older children the ^{90}Sr concentration may in some parts of the skeleton be higher than indicated by the figures for O R (body/diet) It is reasonable to believe that the time required for the establishment of an even distribution of ^{90}Sr at a constant proportion

to calcium i e a maximal value for O R. (body/diet) *is longer than* indicated by the curves in Figure 5 for the older children. An even distribution of ^{90}Sr may never be established under such conditions The reason is that the plasma ratio ^{90}Sr/Ca probably decreases when the absorption decreases and that the elimination in the urine and in the digestive juices increases with age

Figure 6 gives the calculated values for O R. (body/diet) in a young population which has been exposed to a diet containing ^{90}Sr for a period of one two three and four years The O R. (body/diet) in e g a child six years old can be deduced from this Figure after it has consumed the radiostrontium-containing diet for one year (i e from the age of five) etc.

The curves in Figure 6 are based on the same calculations as used in Figure 5 We are now considering the consumption of contaminated diet over a relatively short period in which an even distribution of ^{90}Sr in the skeleton is established only in the youngest children It must therefore be realized that the actual O R (body/diet) in the hot spots is higher than indicated in Figure 6

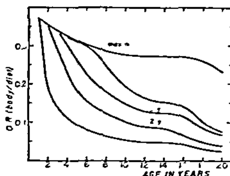


Fig 6 The calculated O R. (body/diet) in a young population after ingestion of ^{90}Sr over a relatively short period.

It is assumed that ^{90}Sr has been ingested at a constant proportion to calcium over a period of time as indicated in the Figure For further details see legend to Figure 5 and the text.

Radiostrontium retention from a diet with a varying ratio of radiostrontium to calcium

The retention of ^{90}Sr from a diet with a constant proportion of ^{90}Sr to Ca represents a simplified imitation of the actual situation following nuclear explosions from which ^{90}Sr is released into the atmosphere The dietary ^{90}Sr will increase to a maximum and next decline if the tests are discontinued It is not possible to calculate a model generally applicable for ^{90}Sr retention under such conditions since the concentrations of ^{90}Sr in the diet in the periods to come are unknown

The results of our method of calculation can be compared with the available figures obtained by ^{90}Sr determinations in human bones (KULP and SCHULERT 1962) Calculations have been carried out using data for the dietary ^{90}Sr concentrations in the eastern United States over the period from 1953 to 1961 According to KULP and SCHULERT (1962) the following figures are representative of the ^{90}Sr concentrations in the region mentioned from 1 July to 30 June each year (in μμc ^{90}Sr per g Ca) 1953–54 1 5 1954–55 3 2 1955–56 4 2 1956–57 6 3 1957–58 10 2 1958–59 16 2 1959–60 14 1 and 1960–61 9 6

These figures were used in calculations in principle identical to those described above (see page 15) Here the fetal content of ^{90}Sr was included and a value of 0 1 was used for O R (fetus/diet)

The results are given in Figure 7 (curve a) Another curve (b) for the expected concentrations of ^{90}Sr in humans living in the same area has been calculated by KULP and SCHULERT (1962) In view of the excellent agreement obtained between the expected and observed values for the skeletal ^{90}Sr concentrations over the previous years curve b probably represents the expected 1961 averages for this particular area

The two curves are comparable in form but our calculations result in the highest values for the ^{90}Sr concentrations A better

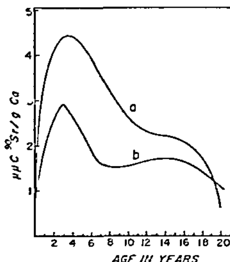


Fig 7 Calculated concentrations of ^{90}Sr in the skeletons of a young population subjected to a diet with a varying proportion of ^{90}Sr to Ca.

Curve a Calculated concentrations of ^{90}Sr in human skeletons for eastern North America at the end of June 1961

Curve b Corresponding curve calculated by KULP and SCHULERT (1962)

It is assumed that the retained ^{90}Sr is evenly distributed in the skeleton. The dietary concentrations of ^{90}Sr used in these calculations are taken from KULP and SCHULERT (1962) these data are found in the text The values used for total body Ca Ca retention Ca intake etc. are found in Table 2 O R. (absorption) is 0 5 O R. (urine/diet) is 1 0 O R. (plasma/diet) is 0.25 and O R. (fetus/diet) is 0 1

agreement between the two curves may be obtained using other values for O R. (absorption) O R. (urine/diet) or digestive juices Ca etc For example when O R. (absorption) is reduced from 0 5 to 0 45 the calculated ^{90}Sr-concentration in the first six years of life is reduced by approximately 40 per cent

In the evaluation of the possible hazards caused by ^{90}Sr–^{90}Y in the skeleton considerable attention should be focused on the protection of the relatively few persons who will achieve the highest skeletal levels of ^{90}Sr The calculated curve (curve a Figure 7) is probably representative of the ^{90}Sr load in such persons and it may therefore serve a useful purpose

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SUBTHRESHOLD DEPOLARIZATIONS IN SKELETAL MUSCLE FIBER DURING LINEARLY RISING CURRENT

STOCKHOLM 1964

CONTENTS

INTRODUCTION

Direct stimulation of skeletal muscle fiber using a current too weak to induce a propagated response elicits a membrane potential change due to the RI drop across the cable network of the fiber (Fatt and Katz 1951, cf Hodgkin and Rushton 1946) It also gives rise to an active subthreshold response or a local response (Kuffler 1942 Jenerick 1956) when the stimulus strength attains about one third or more of the threshold (Katz 1948) this response becomes apparent as a larger depolarization than is to be expected from the cable network

During this response there occurs a change of the AC impedance of the muscle indicative of a rise in conductance although smaller than during a spike (Katz 1942) This rise in conductance has, for several reasons, been ascribed to an increased transfer of sodium ions across the membrane. Thus local depolarizations of low amplitude initiate inward currents which are dependent on the presence of external sodium ions (Strickholm 1962) The active subthreshold response is abolished when the external solution is deprived of sodium, and its appearance is delayed at high concentrations of calcium ions as well as during the influence of local anesthetics (Jenerick 1959 Inoue and Frank 1962) These latter effects have been referred to decrease of the membrane transfer of sodium ions during activation and are consistent with observations made on squid nerve fiber (Frankenhaeuser and Hodgkin 1957 Shanes *et al* 1959 Taylor 1959) These facts, as well as the frequent observation that there is a gradual transition from subthreshold depolarization to spike, indicate that the active subthreshold response is due to activation processes which are not great enough to be regenerative

Previous data on subthreshold processes in muscle fiber have been based on experiments using stimulation with brief electric shocks or longer square wave pulses With both types of stimulation there is usually a progressive increase of the amplitude of the active subthreshold response during the entire period of stimulation It is thus evident that the time required to

each the maximal active subthreshold response may vary with the stimulus duration Scarcely any data are however available concerning slow depolarizations, and none at all from experiments in which the stimulus gradient has been systematically varied Such data should obviously be of importance for a thorough knowledge of the activation properties of the muscle fiber membrane They may also be useful for the understanding of the general mechanism of impulse initiation, in which slow depolarizations are often involved, as has been shown, *e g* during spontaneous activity in muscle fiber (Adrian and Gelfan 1933, Bulbring, Holman and Lullmann 1956, Knutsson not published), in receptor activation (*e g* Bernhard 1942, Katz 1950, Gray and Sato 1953, Ottoson 1956), and during natural activation of spinal neurons (Kolmodin and Skoglund 1958)

When linearly rising currents are applied to frog muscle fiber, the time for depolarization from resting potential to critical membrane potential level can be varied within very wide limits by changing the current gradient (Knutsson and Skoglund 1963) This opens a possibility to study subthreshold phenomena during different depolarization rates and the present investigation was undertaken to see what can be learnt by this new approach

Measurements were made of subthreshold depolarizations in single frog muscle fibers as recorded by an internal electrode in response to linearly rising currents of varying rate of rise applied across the membrane via another internal electrode By means of differentiation, an estimate was also made of the velocity of the membrane potential changes

Since hyperpolarizations were found to be similar to passive chargings of the membrane, they could be used for determinations of the difference between subthreshold depolarization and passive membrane potential change This difference *viz* the active subthreshold response, was determined during stimulating currents of varying gradients It was also determined after prolonged decrease of the membrane potential and after the extracellular fluid had been deprived of sodium ions These studies provide information about the magnitude and velocity of the activation processes In order to determine the spatial distribution of the responses to linearly rising currents intracellular recordings were made simultaneously at different points of a single fiber

METHODS

Isolated sartorius muscles of frog *Rana temporaria* were used The animals were kept at a temperature of 5—15°C During dissection, care was taken to avoid stretching the muscle or increasing its temperature, since many fibers might then exhibit low resting potentials and small spike responses When dissected, the muscle was placed in a bath containing Ringer s solution of the following composition (mM) NaCl 115 KCl 2.5 CaCl 1 8, Na HPO_4 2 15 and NaH_2PO_4 0 85 pH = 7 2 In one series of experiments sodium free solution was used which was made by replacing NaCl by choline chloride on a mole for mole basis

In most of the experiments, and unless special mention is made, the temperature of the bath was kept at 14°C by circulating water from a thermostatically controlled bath through a container surrounding the muscle bath, the temperature variations could thus be kept within a few tenths of a degree

Experiments using two internal electrodes Two microelectrodes one for stimulation and one for recording the membrane potential (cf Knutsson 1961) were inserted into a fiber in an end plate free zone of the muscle (see Katz and Kuffler 1941) The impalements were done under a binocular microscope (40×magnification) the preparation being illuminated from below through the transparent Perspex walls of the muscle bath and the surrounding container The interelectrode distance was measured with an eyepiece scale and kept as close as possible to 30μ the deviation being estimated to be 10% at most

In order to eliminate movement artefacts as far as possible special arrangements were made to ensure that the electrode remained in position during the contraction of the fiber Thus the muscle was placed flat, without any fixation at the bottom of the bath, and very flexible electrodes were used for this purpose capillary electrodes with a long tapering tip were made which could be bent about 2 mm in any direction without breaking The electrodes were mounted on flexible silver wire spirals and inserted at nearly right angles to the surface of the muscle With the aid of

the microscope it was established that the contraction induced by stimulation started in the immediate vicinity of the polarizing electrode and then spread at both sides along the fiber, the two electrodes acting as a fulcrum, at the sides of which contractions occurred (cf. Macfarlane and Meares 1958).

Since movement artefacts may not always be eliminated by this procedure, criteria had to be established to determine acceptable measuring conditions. Movement artefacts due to changes in the position of the recording electrode were easiest to detect since they showed up as abrupt changes of the recorded voltage across the membrane. When momentary voltage shifts caused by movement artefacts exceeded 2 mV, the experiment was discarded. Shifts in the position of the stimulating electrode were more difficult to detect, but in typical cases marked changes could be observed in the relation between the stimulating current and the resultant polarization of the membrane. Such changes are most likely due to a comparatively large current escape when the sealing in of the electrode in the membrane is unsatisfactory. All experiments with an abnormally low or unstable ratio between stimulating current and polarization were thus discarded.

A generator delivering pulses of linearly rising voltage (Haapanen, unpublished) supplied the stimulus. Since its output was in series with a high resistance the rise of the current in the stimulus circuit could be taken as approximately linear. Either positive or negative sawtooth pulses of a maximal voltage of 100 V were obtained. Rates of rise of voltage were continuously variable between 5 and 5 × 10 V/sec. The duration of a pulse could be varied between a few tenths of a msec and 9 sec. The output of the generator was connected to the microelectrode via a series coupled capacitor, 0.05 μF, and a resistor, 50—200 MΩ. The voltage drop across a resistor of either 10 000 or 12,400 Ω was a measure of the current passing the microelectrode and was recorded on one of the beams of a double beam oscilloscope after amplification by a dc amplifier (Grass type P6).

A linearly rising current pulse may sometimes not readily pass the stimulating electrode: this can be seen as a non linear increase of the current flowing through the electrode. In such cases marked voltage changes appear across the recording electrode even if both electrodes are placed in the muscle bath. Under certain recording conditions the stimulating current may thus interfere with the voltage recording and to eliminate such effects all electrodes were submitted to the following test before use. After the two microelectrodes had been placed in the muscle bath 30 μ apart, a linearly rising current was applied to the stimulating electrode and the voltage change across the other electrode was observed. Only when this was insignificant,

i.e. less than about 2 mV, were the recording conditions regarded as satisfactory. This criterion for the selection of the electrodes was, in fact, rigorous since a large number of them might exhibit potential changes of up to tens of millivolts.

The most common reason why an electrode had to be rejected was rectification when a depolarizing current was passed through it. This rectification could be reduced by adding minute amounts of KOH to the KCl filled capillaries and this was also done during one phase of the experiments. When subjected to repeated stimulations such electrodes were, however, apt to deteriorate. Hence, in the later experiments ordinary KCl filled capillaries were employed, and in order to reduce rectification their tips were broken off immediately before insertion into a fiber. For this purpose the electrode was lowered very slowly toward the bottom of the muscle bath under continuous application of stimulating pulses at high repetition rate. As the tip touched bottom, its outermost tip was broken and this was signalled by a change in the current recording. Thus care could be taken to break off only the last tapering portion of the tip just sufficient to permit a linearly rising current to pass. By this means electrodes were obtained which could be used for long series of both outward and inward currents. In some cases, however, a too large portion of the tip was broken off and since this might damage the fiber a final check of the electrode properties had to be made after fiber penetration.

The membrane potential was recorded via an input unit of cathode follower type with a capacitance neutralizing feedback (Haapanen and Ottoson 1954). The output was connected to the second beam of the oscilloscope and to an inkwriter for continuous recording of the membrane potential. The resistance of the recording electrodes varied between 10 and 20 MΩ and their tip junction potentials were less than 5 mV when measured according to the method described by Knutsson (1961). About 90% of the muscle fibers exhibited a resting potential of —88 ± 2 mV, while the remaining fibers varied within a wide range, probably depending on experimental factors. As a rule, the insertion of the stimulating electrode reduced the polarization of the membrane by 1—2 mV. If the membrane polarization during resting state was less than —86 mV when both electrodes were inside the same fiber, the fiber was discarded.

Experiments using three internal electrodes. One electrode was used for intracellular stimulation and the other two for recording of the membrane potential at two different points in one and the same fiber. The stimulating and recording conditions were fundamentally the same as in the experiments with two internal electrodes, but a ganged switch was employed for rapid

shifts between different recordings on the double beam oscilloscope A schematic representation of the stimulating and recording circuits is given in the block diagram Fig 1 With the switch at *c*, stimulating current through electrode S and membrane potential at electrode V_1 were recorded as when using two internal electrodes (cf above) With the switch at *b*, membrane potential was recorded with electrode V_2, stimulus recording being unchanged, whereas with the switch at *a* both V_1 and V_2 recorded the membrane potential During SV_1 and SV recordings the bath was connected to

Fig 1 Block diagram of stimulating and recording circuits used in experiments with three internal electrodes one for stimulation (S) and two for membrane potential recording (V_1 and V) Ganged switch various positions shown separately to the right permitting choice between following circuits

a voltage recording at V and V
b recording of voltage at V and stimulus current through S
c recording of voltage at V and stimulus current through S
d for calibration of V and V circuits
e for compensation of electrode capacitance

ground via a resistor of 10,000 Ω, during V_1V_2 recordings the bath was earthed. Before the electrodes were inserted into the muscle fiber, the different circuits for SV_1, SV_2 and V_1V_2 recordings were balanced. If care was taken to keep a low resistance at the indifferent electrodes, instantaneous shifts between different recordings could be made without the oscilloscope beams being displaced. Two more couplings were used, *viz. c* for adjustment of the capacitance compensation (C-comp.) and employed in order to attain equal responses to a square wave in the two voltage recording circuits, and *d* for calibration of V_1 and V_2 recordings. By attenuating the output signal from one of the cathode followers, equal amplification was obtained for V_1 and V_2 recordings.

The impalement procedure was as follows: *(i)* the V_1 electrode was inserted into the fiber, *(ii)* the stimulating electrode was lowered toward the same fiber under delivery of continuous small stimulating pulses of a frequency of 3/sec., penetration of the membrane was signalled by an electrotonic potential at V_1, thus providing a check that the two electrodes were inside the same fiber, *(iii)* the recording was then switched over to V_1V_2 and the V_2 electrode lowered into the fiber at the required distance from the stimulating electrode. The small stimulating pulses were continuously delivered resulting in electrotonic potentials at V_1 and, as soon as the three electrodes were inside the same fiber, also at V_2. If an electrode slipped out of the fiber this was immediately observed, either as a membrane potential shift or as an absence of electrotonic potentials.

Measurements of the interelectrode distances were usually made by means of the eyepiece of the microscope, but in some experiments the muscle fiber together with the inserted microelectrodes was photographed through the microscope and measurements made on the picture thus obtained, in the latter case more exact calculations could be made on fibers that were slightly bent. Comparisons between these measurements and those performed directly through the microscope showed that the latter could be regarded as accurate within about 10%. All measurements were made in the absence of any induced muscle stretch and before the muscle had contracted in response to a stimulus.

RESULTS

I SUBTHRESHOLD DEPOLARIZATION AND HYPERPOLARIZATION OF MUSCLE FIBER DURING LINEARLY RISING CURRENT

Figure 2 *A* shows a typical example of membrane potential changes in frog muscle fiber (upper trace) in response to a linearly rising current at the rate of rise chosen in this case (lower trace) There is a slow non linear depolarization of the fiber membrane from resting to critical membrane potential, at which a spike is induced (broken line see below) The voltage time curve exhibits a slight upward concavity which becomes more apparent as the depolarization approaches the critical membrane potential level, thus indicating successive changes in the rate of the subthreshold depolarization

Because of the gradual transition from slow subthreshold depolarization to spike potential it is difficult to estimate the exact point on the depolarization curve representing the critical membrane potential level In the present investigations it has been determined by a special procedure which is illustrated in Fig 2 *B* In this record the final phases of the linearly rising stimuli (of the same gradient as in *A*) and the corresponding membrane potential changes are reproduced on a tenfold faster time scale than in *A* In one of the two superimposed recordings the fiber was depolarized to —53 mV during the linearly rising current at that level the current was switched off, resulting in the small downward artefact at arrow 1 in the record The further course of the membrane potential (1) shows a slow repolarization and no spike discharge occurs In the trace marked 2 the current was not switched off until at 2 mV greater depolarization (arrow 2) and in this case a spike develops some milliseconds after break of current The actual level of the critical membrane potential is thus between the two depolarization levels at arrows 1 and 2 but in practice it was defined as the membrane potential at the largest depolarization not resulting in a spike discharge As appears from the broken line connecting records *A* and *B* the critical membrane depolarization is actually slightly lower than the depolarization level at which the spike takes off

Since the critical membrane potential is independent of the stimulus gradient (Knutsson and Skoglund 1963), it was sufficient to make a single determination in each fiber, at an arbitrarily chosen rate of rise of the current, to decide which part of a depolarization should be regarded as subthreshold.

The time courses of a depolarization and a hyperpolarization during linearly rising currents are compared in Fig. 3. Record *A* shows membrane potential changes in response to a linearly rising outward current at an arbitrarily chosen rate of rise (dI/dt) equal to 2.7×10^{-4} A/sec, while record *B* demonstrates a hyperpolarization of the same fiber in response to an inward current of virtually the same duration and gradient -2.6×10^{-4} A/sec (inward current given as negative). As appears from these records there is a gradually increasing upward concavity in the depolarization curve at the end of the subthreshold depolarization similar to that in Fig. 2. In the case of the hyperpolarization there seems to be a practically linear increase of polarization with time and only a barely detectable downward concavity can be

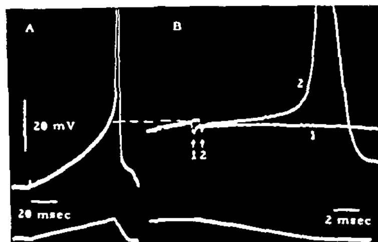


Fig. 2. Membrane potential changes (*upper traces*) in single frog muscle fiber in response to linearly rising current (*lower traces*) applied via an internal electrode across the fiber membrane. Stimulating and recording electrodes 30 μ apart. Resting potential —86 mV.

A. slow depolarization leading to spike discharge during the linearly rising current; rate of rise about 10^{-4} A/sec.

B. to illustrate method of estimation of critical membrane potential level: two superimposed recordings from same fiber at tenfold faster sweep speed showing membrane potential changes during final phase of stimulation using same current gradient as in *A* and current pulses of two different durations. *Trace 1*: current switched off when membrane potential reached —53 mV resulting in small artefact at *arrow 1* followed by slow repolarization (no spike discharge); *trace 2*: current switched off at —51 mV (*arrow 2*) resulting in spike discharge. Broken line indicates critical membrane potential level.

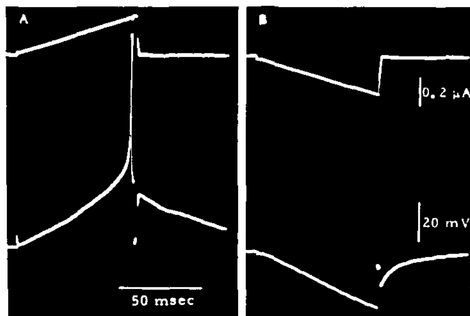


Fig 3 Membrane potential changes *(lower traces)* in single frog muscle fiber resting potential —89 mV during linearly rising current *(upper traces)*

A depolarization during outward current $dI/dt = 2.7 \times 10^{-[illegible]}$ A/sec

B hyperpolarization during inward current $dI/dt = -2.6 \times 10^{-[illegible]}$ A/sec

Stimulating and recording electrodes 30μ apart Capacitative artefacts at onset and end of stimulations

seen in the voltage time curve in the earliest phase of the hyperpolarization

In order to study the course of the polarizations in more detail, the rate of change of the membrane potential was analyzed by graphic differentiation For this purpose, an oscilloscope trace was photographed and its image enlarged and projected onto coordinate paper Straight lines approximately corresponding to tangents to the voltage time curve were drawn through different points of the curve, and their slopes were determined These are assumed to correspond fairly well to the first derivative of membrane potential with respect to time and are given in V/sec Tangents determined in this manner are of course not entirely reliable, but the overall results of a whole series of determinations from a curve representing a relatively slow membrane depolarization or hyperpolarization of the type discussed in this work should give a reasonably good picture of the changes in polarization rate

Figure 4 gives dV/dt of the two records in the experiment Fig. 3. Record A

— corresponding to Fig 3 *A* — shows the rate of the depolarization to the critical potential level, dV/dt is at first about 0 4 V/sec and then increases progressively, attaining 1 7 V/sec at the critical membrane potential level There is thus a successive increase in the rate of the membrane potential change, most pronounced in the final phase of the subthreshold depolarization

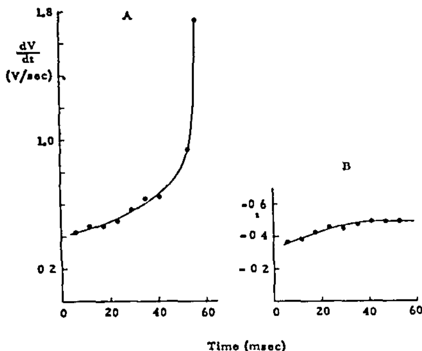


Fig 4 Rate of membrane potential change *(ordinate)* during linearly rising current as estimated from first derivative of voltage change dV/dt obtained from slopes of tangents through different points of voltage time curves *Abscissa* time from onset of stimulation

A during the subthreshold depolarization to critical membrane potential —50mV in Fig 3*A*

B during the hyperpolarization (represented as negative) in Fig 3*B*

In Fig 4 *B*, illustrating the rate of the hyperpolarization shown in Fig 3 *B*, there is a slight initial increase of the numerical value of dV/dt (hyperpolarization given as negative voltage change) resembling the change which occurred during the depolarization After about 30—40 msec, however, dV/dt reaches about —0 5 V/sec and remains constant at that value throughout the stimulation There is thus a slight increase in the rate of the hyperpolarization in the first phase of the linearly rising stimulation, whereas the later

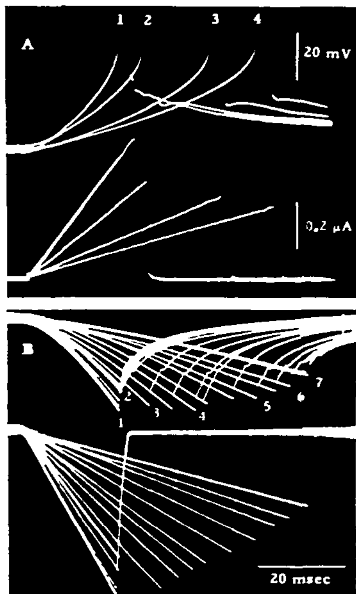


Fig 5 Membrane potential changes (*upper traces*) in single fiber in response to currents of different gradients (*lower traces*) Resting potential —86 mV

A four superimposed depolarizations during outward currents of varying dI/dt (see text) all leading to spike discharge (spikes not shown)

B series of superimposed hyperpolarizations during linearly rising inward current pulses of varying dI/dt see text

The numbers against some of the curves indicate which of them corresponds to each of the

phase exhibits an almost constant hyperpolarization rate. The two graphs clearly show a marked difference between the rate of the subthreshold depolarization and that of the corresponding hyperpolarization, in that during the final phase of the stimulation the former exhibited a marked acceleration while the latter was constant.

Depolarizations at varying dI/dt. Figure 5 illustrates depolarizations and hyperpolarizations in one and the same fiber at different rates of rise of the current. Record *A* shows four depolarizations at $dI/dt = 2.3 \times 10^{-5}$, 1.4×10^{-5}, 7.5×10^{-6} and 5.0×10^{-6} A/sec respectively. Since the adequate stimulus strength for a just subthreshold shock was difficult to determine in advance, the amplitudes of the current pulses were chosen so as always to ensure a spike generation. The four stimulations resulted in slow depolarizations leading to spike discharges 18, 26, 41 and 52 msec, respectively, after onset of the stimuli.

In Fig. 6 *A*, dV/dt during these four depolarizations was plotted against time from stimulus onset, from resting potential, —86 mV, to critical mem-

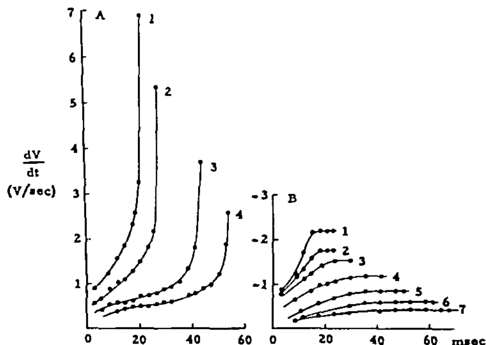


Fig. 6. Experiment of Fig. 5. Change of dV/dt during linearly rising currents *(ordinates)* with time from stimulus onset *(abscissae)*.
A, during the four depolarizations from resting potential, —86 mV, to critical membrane potential, —50 mV, marked 1—4 in Fig. 5 *A*.
B, during the hyperpolarizations marked 1—7 in Fig. 5*B*.

brane potential, —50 mV Curve 1 illustrates the changes in dV/dt during the fastest depolarization shown in Fig 5 *A* in this case the depolarization had reached a velocity of 1 V/sec 5 msec after onset of the stimulus and then rises very rapidly to about 7 V/sec at the critical membrane potential The most marked increase in dV/dt occurs toward the end of the subthreshold depolarization Curve 2 corresponds to the slightly slower depolarization reaching critical membrane potential in 26 msec, in this case the changes in dV/dt are essentially similar but the increase is slower, reaching a maximum value of only about 5 V/sec during the subthreshold depolarization In curves 3 and 4, representing the slowest depolarizations shown in Fig 5 *A* the changes in dV/dt are initially small but increase progressively toward the end of the subthreshold polarizations In the faster of these two curves dV/dt attains about 3 5 V/sec at the critical membrane potential level, while the corresponding value in the slower curve is only about 2 5 V/sec

An extremely slow depolarization is depicted in Fig 7 It was induced by a very slowly rising current (lower trace in *A*) dI/dt being equal to $5\,5 \times 10^{-8}$ A/sec, and resulted in spike discharge 1490 msec after stimulus onset The upper trace in *A* shows that the rate of the depolarization increased substantially during the last 100—200 msec prior to spike discharge A more detailed picture of the depolarization rate is provided by the graph *B* giving dV/dt during depolarization up to 30 mV which was slightly less than the depolarization necessary to attain the critical membrane potential level Determinations of dV/dt at greater depolarizations were however unreliable As indicated in Fig 7 *B* dV/dt was constant at about 0 01 V/sec during the first 700 msec of the stimulation but then gradually rose during the last 800 msec prior to spike discharge, the increase becoming progressively faster toward the end of the stimulation dV/dt reaching a value of about 0 2 V/sec at a depolarization of 30 mV

The changes in depolarization rates illustrated in Fig 6 *A* and 7 *B* are representative of the average depolarization in different frog muscle fibers at different rates of rise of linearly rising stimulating currents This average is based on observations on several hundreds of fibers from about 100 muscles To sum up depolarizations attaining the critical membrane potential level within about 100 msec exhibit a marked progressive rise in the rate of the depolarization throughout the polarization In the case of slower threshold depolarizations of between 100 msec and about half a second there is only a slight increase in the rate of the depolarization in the initial phase of the polarization For still slower depolarizations reaching the critical membrane potential level in about 1 sec the rate of the depolarization is approximately constant during about the first half period of the threshold depolarization

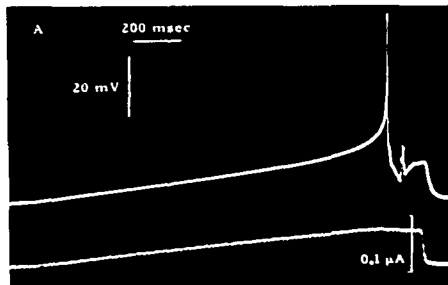


Fig. 7. *A* Extremely slow depolarization *(upper trace)* during linearly rising current $dI/dt = 5.5 \times 10^{-8}$ A/sec *(lower trace)* leading to spike discharge 1490 msec after stimulus onset.
B dV/dt during this depolarization plotted against time from stimulus onset. dV/dt attained ca. 0.2 V/sec at 30 mV depolarization but is followed only to 0.1 V/sec in the curve.

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of up to 20—30 mV At hyperpolarizations exceeding about 30 mV gradual reductions of the numerical value of dV/dt were regularly observed following upon the periods of constant dV/dt thus indicating progressive reductions in the hyperpolarization rates

Comparisons between subthreshold depolarizations and hyperpolarizations in response to currents of about equal strength but of opposite direction (see Fig 3 and 5) show that the potential changes are similar in the initial phases of the polarizations whereas the final phases always exhibit pronounced differences During a depolarization there is thus as the critical membrane potential is approached, an acceleration of the depolarization irrespective of the current gradient applied whereas in the final phase of a hyperpolarization the increase in the rate of the membrane potential change slows down and a constant rate of polarization is attained

This difference between a hyper and a depolarization *i.e.* the accelerating increase of a subthreshold depolarization, is supposed to be due to changes in the fiber membrane occurring in the course of the depolarization In the following the term activation will be adopted for these changes (cf Shanes 1958, Jenerick 1959)

II TIME COURSE OF CHARGE OF PASSIVE PARALLEL RC NETWORK FED FROM A RAMP FUNCTION GENERATOR — MODEL EXPERIMENTS

In view of the rather complex phenomena connected with polarization induced by currents of different rates of rise it is difficult to determine to what extent the polarization is due to passive properties of the fiber membrane and to what extent it may be ascribed to changes taking place in the membrane in the course of the polarization *i.e.* altered membrane constants or current generation To understand how a polarization observed in a membrane has originated it would be of interest to know how it would have developed if the membrane had behaved strictly passively To some extent such information can be obtained by studying the time course of the charging when a parallel resistance capacitance network is fed from a generator of linearly rising current

For this purpose model experiments were performed in which currents of various rates of rise were applied across a circuit of this type, and the resultant voltage (V_{RC}) across the parallel RC network was recorded (see Fig 8) In one series of experiments the time constant was varied between 10 and 80 msec by varying the value of C while the value of R was kept constant

at 0 05, 0 2 or 1 0 MΩ, in another series the time constant was kept constant at 35 msec while the ratio R/C was varied

Figure 9 illustrates the charging of the RC network during linearly rising currents, the resistance being 0 2 MΩ parallel with a capacitance of 0 175

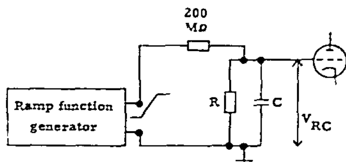


Fig 8 Arrangement for measuring charging (V_{RC}) of passive parallel RC network

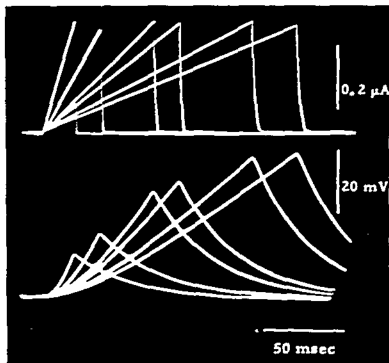


Fig 9 Charging of passive parallel RC network during linearly rising current Superimposed records of current pulses (*upper traces*) and concomitant voltage changes (*lower traces*) R 0 2 MΩ C 0 175 μF Full explanation in text

μF The values of R and C were chosen so as to give a time constant of 35 msec (thus of the same value as the time constant in frog muscle according to Fatt and Katz 1951), and so as to give a value of V_{rc} about equal to the polarization across the fiber membrane during a linearly rising current From the superimposed recordings of V_{RC} using values of dI/dt varying between 2.1×10^{-5} and 2.0×10^{-4} A/sec it appears that during a linearly rising current across the RC circuit the time course of V_{rc} is, in each case at first non linear and then becomes approximately linear It can also be seen that V_{RC} at a certain current strength is larger, the lower the rate of rise of current

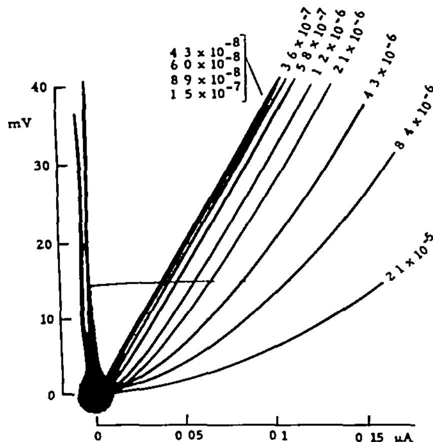


Fig 10 Direct recordings of voltage-current curves during linearly rising current Oscilloscope spot tracing the charge of the passive parallel RC network *(ordinate)* as a function of the linearly rising current *(abscissa)* Series of superimposed records at different rates of rise of the current Four coinciding recordings *(steepest curve)* were obtained at the four current gradients indicated to left of curve other current gradients given against each appropriate curve Vertical traces to left as well as thin horizontal line oscilloscope spot returning to zero R = 0.2 MΩ C = 0 175 μF

Thus, e g, during the fastest rise of the current to 0 3 μA V_{RC} reaches a maximum value of only 11 mV, whereas during the slowest rise to the same current strength V_{RC} attains a maximum value of 40 mV

If the rate of rise of the current is sufficiently low, the time constant can be expected to have practically no effect on the charge of the *RC* circuit The highest value of dI/dt necessary to obtain this result was measured at different time constants For this purpose the ratio V_{RC}/I during linearly rising currents of varying dI/dt was directly recorded using the X Y display feature of

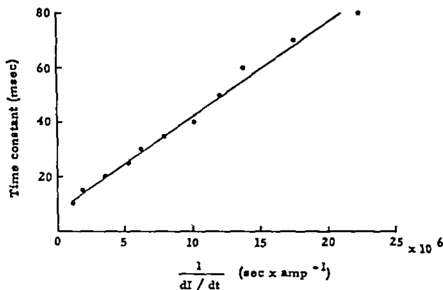


Fig 11 Reciprocals of critical dI/dt *(abscissa)* at different time constants *(ordinate)* in a passive RC network R=0 2 MΩ C varied to give time constants from 10 to 80 msec Critical dI/dt determined by direct recording of V/I curves and taken as the largest dI/dt at which the influence of the time constant was negligible

the oscilloscope Figure 10 illustrates such recordings from an experiment in which the rate of rise of the current was varied between 4 3 $\times 10^{-8}$ and 2 1 $\times 10^{-5}$ A/sec The lowest right hand curve represents V_{RC}/I at the steepest current gradient while the other curves show V_{RC}/I at stepwise diminishing values of dI/dt When dI/dt is reduced V_{RC}/I gradually rises until it reaches a constant value equal to *R* in the *RC* circuit This was attained at a value of dI/dt equal to 1 5 $\times 10^{-}$ A/sec, corresponding to the highest rate of rise at which the time constant is negligible This value of dI/dt will in the following be termed the *critical* dI/dt

The reciprocal of critical dI/dt at various time constants has been plotted in Fig 11 By changing C the time constant in the RC circuit was varied between 10 and 80 msec which covers the variations in frog muscle fiber membranes *i.e.* about 20—40 msec (Fatt and Katz 1951 del Castillo and Machne 1953) The resistance was kept constant at 0 2 MΩ, this value being chosen as being of the same order as the effective resistance in frog muscle fibers

The values obtained in the measurements Fig 11 show that there is an approximately linear relationship between the time constant and the reciprocal of critical dI/dt This implies that as was to be expected critical dI/dt is reduced as the time constant increases The data given in Fig 11 can consequently be employed for an approximate determination of the steepest gradient of a linearly rising current at which there is no time constant effect on the charge

The relationship between time constant and critical dI/dt in the RC circuit was studied also at resistances other than 0 2 MΩ, and it was established that the ratio was not significantly altered when R was varied between 0 05 and 1 0 MΩ

The charge across the RC circuit during linearly rising currents closely resembles the hyperpolarizations in muscle fiber membranes, as appears from a comparison between Fig 5 B and Fig 9 The similarities thus observed support the assumption that the non linearity of the hyperpolarizations is caused by the time constant of the membrane Passive polarization could thus sufficiently explain the course of hyperpolarizations less than 20—30 mV However the similarities between charging of a passive RC circuit and hyperpolarizations of the fiber membrane do not of course exclude changes in the membrane constants of a fiber during hyperpolarization since slow or small changes in the membrane are not likely to have any perceptible effect on the time course of a polarization

III "PASSIVE" AND "ACTIVE" MEMBRANE POTENTIAL CHANGES IN RESPONSE TO LINEARLY RISING CURRENTS

The relatively large time constant of the muscle fiber membrane evidently plays a decisive role for the time course of polarizations during application of linearly rising currents across the membrane As soon as the rate of rise of the current is larger than the critical dI/dt (see section II), the membrane potential change is invariably delayed by the effect of the time constant and the result is thus not a simple RI drop across the effective resistance of the mem

brane This time constant effect varies with the current gradient and can be expected to be progressively more pronounced as the rate of rise of the current is increased Since the magnitude of this effect at a certain current gradient cannot readily be calculated, all attempts to distinguish passive membrane potential changes from those caused by changes in the membrane properties will thus meet with difficulties

If the resting membrane could be assumed to be similar to a distributed, parallel resistance capacitance line it would be possible to determine to what extent a subthreshold depolarization is due to activation' processes in the membrane, by applying a method analogous to that used by Jenerick (1959) in his studies of responses obtained by square wave stimulation of muscle The magnitude of a passive polarization could then be calculated and compared with the actual depolarization observed This would require determinations of membrane resistance and time constant as well as calculations of the effect of the time constant at the rate of rise of the current actually applied to the fiber under study Such calculations would be uncertain and the final results rather unreliable besides the method would introduce considerable complications in the experimental procedure

Another method of studying subthreshold activation processes would be to compare a depolarization during a linear stimulating current with a hyperpolarization produced by a current of equal strength but of opposite direction (cf Katz 1948 see also Hodgkin 1948) This would be a fairly practicable method provided that such a hyperpolarization did not give rise to any appreciable changes in the membrane constants for the hyperpolarization observed would then be approximately equal to a passive polarization and no determinations of membrane resistance time constant or the effect of the latter on the polarization would be necessary However hyperpolarizations do in fact give rise to certain changes in the membrane constants thus the AC impedance in muscle varies continuously with the strength of the polarizing current (Dubuisson 1935) and according to Adrian and Freygang (1962) the membrane conductance in individual frog muscle fibers is altered during a hyperpolarizing square wave stimulation During anodal stimulation of threshold strength there is an increase of about 0 1% in the AC impedance in sartorius muscle (Katz 1942) This seems to correspond to a rather small change in conductance The question then arises whether it is large enough to exert any significant effect on the polarizations This seems actually not to be the case according to earlier investigations showing that the voltage-current relation is linear during steady state hyperpolarizations using square wave pulses up to about threshold strength (Katz 1948 Boyd and Martin 1959) and during slow continuously rising outward

currents (Hutter and Padsha 1959, Knutsson 1961) This view is further supported by the results of the experiments to be described below

Similarities between hyperpolarizations and passive polarizations

Since the conductance in muscle fiber membrane is likely to increase during depolarization and diminish during hyperpolarization (Dubuisson 1935, Katz 1942), one would expect that currents of opposite direction would give rise to polarizations of different magnitude if the conductance changes were large enough to have any perceptible effect When, however, hyperpolarizations and depolarizations are produced by currents of the same rate of rise but of opposite direction (cf Fig 5), the resultant potential changes are equal up to 5—20 mV (the highest values obtained during rapid polarizations) and it thus seems reasonable to assume that the conductance changes during these relatively small polarizations are too insignificant to have any influence on the polarizations

In one series of experiments the voltage current ratio across the membrane was determined by application of small linearly rising current pulses and simultaneous recording of the resultant membrane potential changes Variations in the effective resistance or in the time constant of the membrane would then appear as a change in the voltage current ratio When the membrane potential was preset at hyperpolarized levels up to about 30 mV above normal this ratio was found to remain unchanged if any changes in the membrane constants were produced by the hyperpolarizations they were too small to be perceived

In another series of experiments critical dI/dt was determined *viz* the largest current gradient at which the time constant has no effect on the polarization (cf model experiments section II) Such determinations give an estimate of the time constant of the membrane during a hyperpolarization with linearly rising current and the results obtained can thus be compared to earlier calculations based on responses to small hyperpolarizing square wave pulses A typical experiment is illustrated in Fig 12, in which the relation between membrane potential change and current (V/I) during hyperpolarizations was plotted against dI/dt Since V/I varies in the course of one and the same polarization a specific value has to be chosen, and in this case the value attained at a hyperpolarization of 15 mV was given as V_{15}/I As appears from the figure V_{15}/I is constant when dI/dt is below a certain value $5 \times 10^{-}$ A/sec which is critical dI/dt for this fiber By introducing the value of the reciprocal of critical dI/dt in the relation given in Fig 11, a measure of the time constant was obtained in this case 15 msec In similar determinations

on a series of different fibers, time constant values between 15 and 45 msec were observed These results can be regarded as reasonably consistent with previous calculations of the time constant in frog muscle fiber during square wave stimulation (Fatt and Katz 1951, del Castillo and Machne 1953), thus indicating that the time constants observed during a linearly rising hyper polarization do not appreciably differ from those obtained during stimulation with low amplitude, hyperpolarizing square wave pulses, during which the membrane may be regarded as a passive conductor

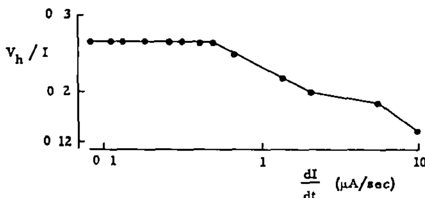


Fig 12 Voltage current ratio (*ordinate*) during linearly rising hyperpolarizing current of varying rate of rise (*abscissa*) V_h/I determined in single fiber at 15 mV hyperpolarization (voltage in μV and current in ampere)

Although changes in membrane conductance may be supposed to occur during a hyperpolarization, there is strong evidence in support of the assump tion that a hyperpolarizing current of threshold strength, resulting in hyper-polarizations of 20—30 mV is small enough to keep the membrane within a linear part of its characteristics It would thus appear justifiable to regard a hyperpolarization of less than 20—30 mV as an approximate measure of a passive change of membrane potential consequently, the method described below was applied to distinguish between active and passive phases of sub threshold depolarizations

Determinations of active subthreshold potential A depolarizing linearly rising stimulating current pulse strong enough to give rise to a spike discharge was first applied After reversal of the current hyperpolarizing stimuli of about the same gradients as the depolarizing currents were applied The in verted trace of the hyperpolarization thus produced by a current of the same rate of rise as the depolarizing current was superimposed on the trace ob

tained from the depolarization The procedure is illustrated in Fig 13, from which it appears that the two curves coincide in the initial stages of the polarizations but begin to separate at a depolarization of 10 mV The membrane potential level at that stage of depolarization will be termed *activation potential* in analogy with the nomenclature used by Jenerick (1959) The potential difference between the two curves up to the moment when the critical membrane potential is attained is called *active subthreshold potential,* ASP (cf Shanes 1958) This difference exhibits a successive increase with time, in this case up to 10 mV at the critical membrane potential level 29 msec after the separation point of the curves

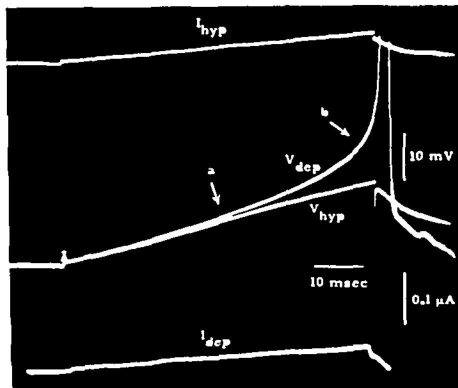


Fig 13 To illustrate determination of active subthreshold potential Depolarizing current pulse (I_{dep}) gives rise to potential change (V_{dep}) leading to spike discharge onset of which is apparent as upward deflection at end of depolarization I_{hyp} and V_{hyp} are superimposed inverted records of current pulse of the same gradient as I_{dep} and potential change during hyperpolarization Arrow *a* indicates activation potential arrow *b* approximate critical membrane potential the difference between the two records from *a* to *b* constitutes ASP

Activation potential A typical example of determinations of active subthreshold potentials is given in Fig 14 in which records *A*—*L* show the time courses of depolarizations leading to spike discharges, in each record at a different gradient of the stimulating current, the superimposed tracings in *F* and *G* show the time courses of hyperpolarizations at different gradients Five of these tracings each corresponding with respect to gradient to one of the depolarizations in *A*—*E* have been inverted and are represented by the dotted lines in the depolarization records The difference between the depolarization curve and the dotted line thus represents ASP

The activation potentials in records *A*—*E* are indicated by small gaps in the depolarization curves The depolarization necessary to reach the activation potential is 17 mV in record *A* during the fastest depolarization as the rate of rise of the stimulus is reduced it becomes successively smaller, being 13, 8, 9 and 6 mV in *B*, *C* *D* and *E* respectively The resting potential was —88 mV, and the activation potential thus varies within a fairly wide range with the rate of rise of the stimulating current as is also evident from Table I, showing the activation potential values obtained in this experiment The values indicated in the table are fairly typical of the actual changes in activation potential with variations of the depolarization rate observed in other experiments The absolute values of the activation potential may, however vary somewhat in different fibers Typical values during a depolarization to critical membrane potential in 10—20 msec are —65 to —72 mV During slower depolarizations leading to spike generation in 500—1500 msec values of —75 to —82 mV are obtained

According to Fig 2 in Jenerick s paper of 1959 the activation potential in muscle fibers with resting potentials between —85 and —90 mV varied between about —65 and —70 mV This corresponds fairly well to the activation potential at the fastest depolarizations produced by a linearly rising

TABLE I Activation potential during different dI/dt in single muscle fiber Experiment of Fig 14 Resting potential —88 mV Temperature 14 C

dI/dt (A/sec)	Spike latency (msec)	Activation potential (mV)
5 6 × 10[illegible]	26	—71
2 2 × 10[illegible]	53	—75
8 0 × 10[illegible]	110	—80
4 [illegible] × 10[illegible]	195	—79
1 9 × 10[illegible]	370	—82

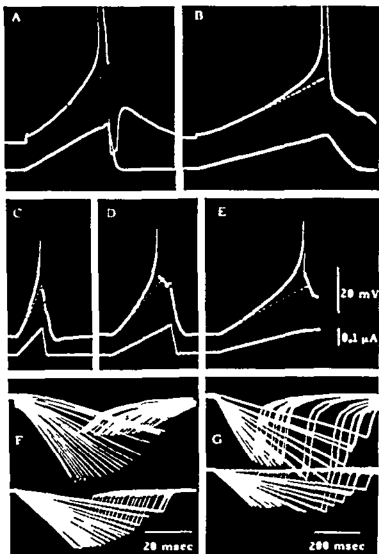


Fig 14 Records illustrating determinations of ASP in single muscle fiber at five different current gradients *Upper traces* membrane potential changes *lower traces* current pulses *A—E* depolarizations leading to spike discharges (spikes not shown) *F* and *G* superimposed hyperpolarizations at different current gradients those corresponding to the gradients in *A—E* respectively being shown inverted in these curves *(dotted lines)* Time bar in *F* also refers to *A* and *B* that in *G* also to *C—F* Current and potential calibrations in *E* apply to all records Resting potential —88 mV

current pulse, and there is thus good agreement between the activation potential obtained by square wave stimulation of frog muscle fiber and that resulting from rapid depolarizations using linearly rising currents However, *the definition of an activation potential given by Jenerick differs* somewhat from that applied in the present work, since he measures the onset of activation at the separation point between a depolarization and a passive polarization calculated by extrapolation from small responses obtained by depolarizing square wave pulses In the present investigation, on the other hand, onset of activation has been measured with reference to a passive hyperpolarization

Several sources of error have to be considered in determinations of the onset of ASP In the first place, the difference between depolarization and hyperpolarization must amount to about 0 5 mV to be detectable Besides errors of a technical nature such as the thickness of the oscillograph tracing on the photographic film small disturbances in the recordings and optic deviations in the projection procedure have to be taken into account Since the membrane potential changes occur relatively rapidly a slight inaccuracy in the measurement of the separation point between depolarization and hyperpolarization may introduce a serious error in the estimate of the activation potential The value of a single determination should thus be regarded as accurate only within a few millivolts One may, however safely conclude that signs of activation processes appear at a smaller depolarization during slower than during faster depolarizations In absolute values the differences obtained at varying rates of depolarization may however vary somewhat in individual fibers

Increase of ASP with depolarization In order to determine the relation between ASP and membrane potential during depolarizations induced by linearly rising currents, the ratio between remaining voltage across the membrane during a depolarization and the active subthreshold potential was studied Figure 15 shows typical results obtained in a single fiber at four different rates of rise of the current as appears from the figure the active subthreshold potential corresponding to each membrane potential value is larger at slow than at rapid rates of depolarization Thus ASP reached 16 mV in the course of the slowest depolarization just before the critical membrane potential level, —52 mV was attained, whereas it had a value of only 7 mV at the fastest depolarization to this critical potential The active subthreshold potential is thus apparently not solely a function of the membrane potential *but is also to a large extent dependent on the rate of the depolarization*

Activation processes in muscle fiber have previously been studied by means of square wave stimulation which only to a limited extent permits variations

of the rate of depolarization When the relation between ASP and membrane potential is studied only during stimulation by a relatively rapidly rising current (e g 5 6×10⁻ V/sec, see the lowest curve in Fig 15), the active subthreshold potential actually varies almost linearly with the membrane potential (cf Jenerick 1959) and it may then seem relevant to draw the conclusion that this observation should have general validity Since however the active subthreshold potential has now been shown not to be entirely dependent on the membrane potential but also on the rate of the membrane potential changes as described in this work, some supplementary information has thus been obtained about the subthreshold activation properties

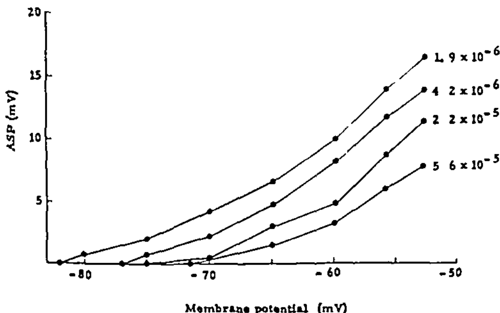


Fig 15 Relation between ASP and membrane potential in single fiber during depolarizations induced by linearly rising currents of different gradients (indicated to the right of each curve)

Rate of rise of ASP In Fig 16 the active subthreshold potential in a fiber at an arbitrarily chosen rate of rise of the current 3 4×10⁻⁶ A/sec has been plotted on a logarithmic scale against time from its onset i.e. from the moment at which the depolarization and the hyperpolarization can first be seen to separate In this experiment the onset occurred when the depolarization had reached 16 mV The resting potential in the fiber was —88 mV, and the activation potential was thus —72 mV During the further course of the de

current pulse, and there is thus good agreement between the activation potential obtained by square wave stimulation of frog muscle fiber and that resulting from rapid depolarizations using linearly rising currents However, the definition of an activation potential given by Jenerick differs somewhat from that applied in the present work, since he measures the onset of activation at the separation point between a depolarization and a passive polarization calculated by extrapolation from small responses obtained by depolarizing square wave pulses In the present investigation, on the other hand, onset of activation has been measured with reference to a passive hyperpolarization

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Activation processes in muscle fiber have previously been studied by means of square wave stimulation which only to a limited extent permits variations

(record *A*), whereas it takes about 260 msec to reach a value of about 15 mV during the lowest rate of rise of the current (record *E*). The question then arises in what manner the rise of ASP varies with the rate of rise of the stimulus current, and this is illustrated in Fig. 17, in which the active subthreshold potentials obtained during the five depolarizations shown in Fig. 14 have been plotted against time from onset of ASP. The semilogarithmic plottings follow reasonably well the straight lines marked *a—e*, corresponding to the active subthreshold potentials in Fig. 14, records *A—E* respectively. As the stimulus gradient is reduced, the slope of the lines becomes less steep. There is thus a diminution of the rate of rise of ASP as the rate of rise of the stimulus is diminished.

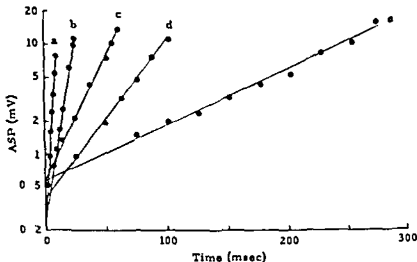


Fig. 17. Experiment of Fig. 14. Active subthreshold potentials (logarithmic scale) plotted against time from onset of ASP. Points correspond to measurements of difference between depolarization and hyperpolarization at the five current gradients given in Table II, the points along the straight lines *a—e* corresponding to the active subthreshold potentials in records *A—E* respectively in Fig. 14.

To facilitate more conclusive comparisons between the active subthreshold potentials obtained at different stimulus gradients, ASP may be expressed by the equation

$$\mathrm{ASP} = A \times \exp(k \times t) \quad (1)$$

where t is time from onset of ASP, and A and k are constants. After plotting ASP on a logarithmic scale against time, the straight line on which the

experimental points fall can be used for determining A and k, A from the point at which the line intersects the vertical axis ($t = 0$) and k from the slope of the line, $1/k$ being the time during which ASP increases e times In the calculations ASP has been expressed in volts and time in seconds

The values of the constants A and k as calculated from the experiment in Fig 14 and 17 are given in Table II In this experiment the value of A varied between $2\,2 \times 10^{-4}$ and $5\,7 \times 10^{-4}$ This corresponds approximately to the smallest discernible difference between hyperpolarization and depolarization (in volt) The fact that the largest values of A were obtained at

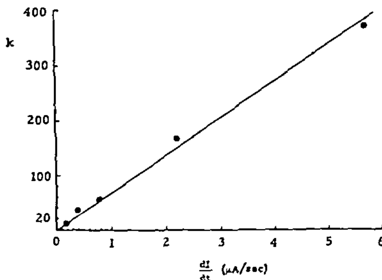


Fig 18 To illustrate changes in the rates of the active subthreshold depolarization with the gradient of the stimulus current. Rate constant k plotted against rate of rise of stimulus current as determined in single fiber Values of k and dI/dt cf Table II

the lowest rate of rise of the stimulating current may be explained by the very slow sweep speed used in these recordings since this made it more difficult to observe the divergence between hyper and depolarization

The value of the constant k, which gives an expression of the rate of the exponential rise of ASP, varied in this experiment between 379 and 12 In the case of the fastest depolarization in Fig 14, k was thus about 30 times larger than in the slowest one There is thus a considerable variation of k larger than the variation in time observed for depolarizations from resting potential to critical membrane potential, *viz* between 26 and 370 msec

In order to study the changes in the rate of the active subthreshold de

polarization with the gradient of the stimulus current I was plotted against dI/dt (Fig 18), the result is an approximately linear relation Thus k seems to be virtually proportional to the gradient of the stimulating current Similar results were obtained in practically all fibers tested the few exceptions observed were probably due to technical errors It can thus be established that the rate of the exponential rise of ASP varies with the gradient of the stimulating current in a characteristic and regular manner

TABLE II Numerical values of constants A and k in single muscle fiber during different dI/dt Experiment of Fig 14 Resting potential 83 mV Temperature 14 C

	dI/dt (A/sec)	A	k	k dI/dt
A	5 6×10⁻	2 2×10	3?9	6 8×10
B	2 2×10⁻⁴	2 1×10	167	7×10
C	8 0×10⁻	2 8×10	549	7 6×10
D	4 2×10⁻	4 0×10	345	8 3×10
E	1 9×10⁻³	5 7×10⁻	1?0	6 4×10
			Mean	4×10

Concomitantly with the gradually diminishing slopes of the lines a—e in Fig 17 there are changes of the maximum value of the active subthreshold potential attained at the moment when the depolarization reaches the critical membrane potential level This value is 8 10 13 11 and 16 mV in a b c d and e respectively There is thus except in d a successive increase with diminishing slope, and this was a regularly observed event in practically all experiments Whether the exception to this rule observed in d is reproducible cannot be decided from the present evidence it may well have been due to some experimental factor It thus seemed justified to draw the conclusion that ASP usually attains progressively higher maximal amplitudes as its rate of rise is reduced

Direct comparisons of ASP in different fibers have to be made at equal stimulus gradients since ASP varies with dI/dt In practice however, such comparisons cannot be made because a repetition of a particular rate of rise of current is not possible when stimulating different fibers Instead the ratio k dI/dt in different cases may be compared since this is reasonably constant in an individual fiber as was apparent from the linear relation between k and dI/dt in Fig 18 and is also evident from the values of k dI/dt in the same experiment given in the right hand column of Table II The mean value of k dI/dt at the five different rates of rise of the stimulus current was 7 4×10 , range 6 4×10 to 8 3×10 Thus a new constant $k_{ASI} = k$ dI/dt,

may be introduced, and when substituting k in equation (1) the following equation is obtained

$$\mathrm{ASP} = A \times \exp(k_{\mathrm{ASI}} \times \mathrm{d}I/\mathrm{d}t \times t) \qquad (2)$$

This equation can be applied to experimental results irrespective of the rate of rise used By comparisons of k_{ASI}, the variations in the rise of the active subthreshold potential in different fibers may thus be determined

The values of k_{ASI} in a typical series of experiments at room temperature appear from Table III In these eight fibers, the mean value was $2\,2 \times 10^{7}$, range $1\,5 \times 10$ to $3\,4 \times 10^{7}$ This is no large variation, considering possible variations due to differences in fiber size and errors involved in the deter minations, and it is possible that they reflect only minor variations in sub threshold excitability properties It should however be noted that, in some series of experiments, k_{ASI} in a few fibers exhibited marked deviations from the values obtained in the majority of the fibers These deviations were too irregular to be accessible to a closer analysis, and they may indicate that a few fibers differ significantly with respect to the rate of the subthreshold activation There may also be differences in the values of k_{ASI} at different temperatures, as appears from a comparison of the results listed in Tables II and III obtained at 14 and about 22°C respectively The effects of tempera ture on ASP were however not systematically studied in the present investiga tions, and the divergences may have been due to other factors

Since it is well known that activation processes in muscle fiber are influ enced by depolarizations it might be suspected that a prolonged decline of the resting potential may alter the rate of rise of the subthreshold potential In order to explore if the rates of the subthreshold activation processes are in fluenced by sustained depolarization, k_{ASI} was determined after presetting

TABLE III Numerical values of constants k and k_{ASI} in eight different muscle fibers during different dI/dt Temperature 21 – 23 C

Fibe	dI/dt (A/)	k	k_{ASI}
1	2 9×10⁻	50 1	1 7×10
2	4 1×10⁻	138 8	3 4×10
3	8 7×10⁻	183 7	2 1×10
4	8 3×10	216 8	2 6×10
5	5 6×10⁻	143 3	2 5×10
6	5 4×10⁻	81 5	1 5×10
7	3 1×10⁻	56 9	1 8×10
8	1 6×10⁻	36 7	2 3×10⁷
			Mean 2 2×10

the membrane potential at varying levels. The result is illustrated in Fig. 19 in which k_{ASI} has been plotted against membrane potential immediately prior to onset of a linearly rising stimulus. The variations in membrane potential were either caused by "spontaneous" depolarizations in the fibers under study or induced by polarizing constant current. As a rule only one or two different rates of rise of the stimulus were applied in a single fiber since the variations of k_{ASI} at different current gradients in a particular cell were insignificant. As appears from the figure, there is a tendency toward increasing values of k_{ASI} as the membrane potential is reduced, indicating that the rate of active subthreshold depolarization is increased in fibers which have been slightly depolarized.

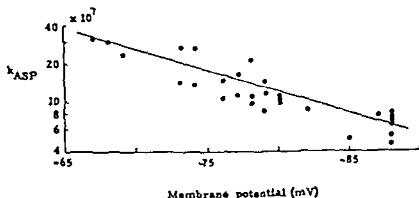


Fig. 19. Constant k_{ASI} (logarithmic scale) plotted against membrane potential immediately prior to onset of linearly rising current pulses. Membrane potential variations due to spontaneous depolarization or induced by polarizing current. Line drawn by free hand.

Sodium free solution

Twelve fibers were tested in a solution in which the Na ions were replaced by choline. A typical result is illustrated in Fig. 20 showing the depolarizations at four different current gradients successively reduced in records *A* to *D*. The time courses of the depolarizations are similar to passive discharges of the membrane capacity during the first phase of a stimulation until the depolarization reaches about 40, 33, 28 and 22 mV in *A*—*D* respectively. During the further course of the depolarizations there is a successive decrease of the depolarization rate, *i.e.* a rectification, which in *A* abruptly increases at a depolarization of 53 mV, while the rectification in *B* and *C* is less

rupt, the depolarization reaching a plateau at 45 and 35 mV respectively. In *C* the rectification increases slowly during the stimulation which was discontinued when the depolarization had reached 28 mV. It is obvious from these recordings that the active subthreshold potential is abolished in the sodium free solution, which is in good agreement with previous observations (see, *e g*, Jenerick 1959). It also becomes apparent that the rectification, which has been ascribed to increased membrane conductance to potassium ions, varies with the stimulus gradient both with respect to onset and time course. Thus, onset of rectification is discernible at a smaller membrane potential change the slower the depolarization. The rate of its increase, *viz* the

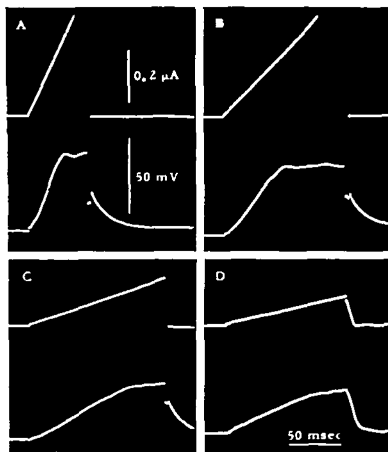


Fig 20. Membrane depolarizations (*lower traces*) in single frog muscle fiber in sodium free solution in response to linearly rising current (*upper traces*) of four different in records *A*—*D* successively reduced gradients. Resting potential —87 mV. Calibrations in *A* and *D* apply to all records.

rate of change of the voltage current relation is also less the slower the depolarization

The changes in onset and rate of the rectifying process at varying current gradients are thus similar to the changes of the active subthreshold potential at varying current gradients However the observations made when using sodium free solution showed rather marked quantitative variations in different fibers and could thus not be submitted to a closer analysis

IV SITE OF ORIGIN AND SPREAD OF RESPONSES TO SLOWLY RISING CURRENTS

In order to explore in what part of the muscle fiber the responses to a linearly rising stimulating current originate and how they spread simultaneous recordings were done of membrane potential changes using two internal electrodes placed at various distances from the stimulating electrode Thirty three fibers from fifteen muscles were examined The arrangement of the electrodes is shown in the schematic drawing at the top of Fig 21 The distance (x) between the stimulating electrode (S) and the nearest recording electrode (V_1) ranged between 15 and 1620μ while the distance (y) between the stimulating electrode and the more distant recording electrode (V_2) was between 80 and 3560μ At these electrode positions the distances between electrodes V_1 and V_2 had a range of variation of 30—1940μ Separate descriptions of the subthreshold responses and the propagated responses thus obtained will be given below

Subthreshold responses A typical experiment is illustrated in Fig 21 The stimulating pulses through electrode S are identical in records *A* and *B* and the concomitant membrane potential changes in *A* are recorded by electrode V_1 located 160μ, and in *B* by electrode V_2 940μ from the stimulating electrode As appears from the two records the amplitude of the slow membrane potential change preceding the spike is lower in *B* than in *A* this is still more conspicuous in record *C* showing simultaneous recordings of the responses obtained at V_1 and V_2

In Fig 22 *A* the depolarization at electrode V_2 in per cent of that at V_1 has been plotted at different times from onset of the linearly rising stimulus After about 25 msec the change at V_2 amounts to about 50% of that observed at V_1 and this value then remains practically constant up to spike discharge The difference between V_1 and V_2 is thus most pronounced in the initial phase of the depolarization and then gradually declines to a constant final value

Figure 22 *B* shows the results of similar determinations on another fiber, electrode V_1 being located at a distance of 80μ, and V at a distance of 1380μ, from the stimulating electrode The resulting curve resembles that in *A* but in this case a constant level of about 70% is reached in the final phase of the subthreshold depolarization, the polarization in the last phase of the stimulation thus declining only about 30% along a fiber length of 1300μ

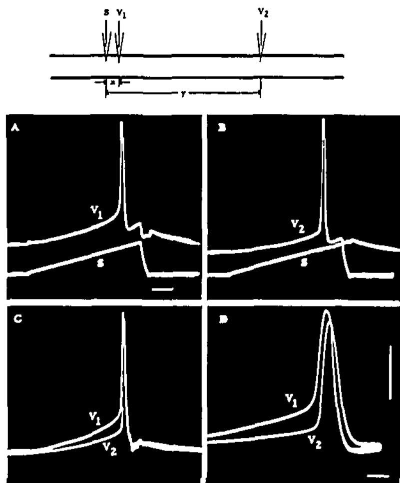


Fig 21 Membrane potential changes at different distances from site of st response to linearly rising current Diagram at top of records shows electrode V and V_2 representing the recording electrode 160μ (x) and 910μ (y) the stimulating electrode (S) Records *A* and *B* identical current pulses n tential changes in *A* recorded by V and in *B* by V_2 *C* superimposed obtained at V and V *D* same as in *C* but on faster time scale showing onl of stimulation Time bar in *A* 10 msec also refers to *B* and *C* Time bar in *D* age calibration in *D* 50 mV applies to all records

Figure 22 *C* illustrates the differences in subthreshold membrane potential changes along a muscle fiber as compiled from data collected in 10 experiments For these determinations the levels attained about 5 msec prior to spike discharge were selected, since the decline of a depolarization along a fiber in per cent was practically constant during the later part of a subthreshold depolarization The depolarization at V_1 and the simultaneous depolarization at V were plotted on a logarithmic scale against distance from the stimulating electrode Straight lines were drawn through the points representing the two values obtained from each individual fiber The slopes of these lines are comparatively similar and give a measure of the amplitude reductions of the subthreshold membrane potential changes along a muscle

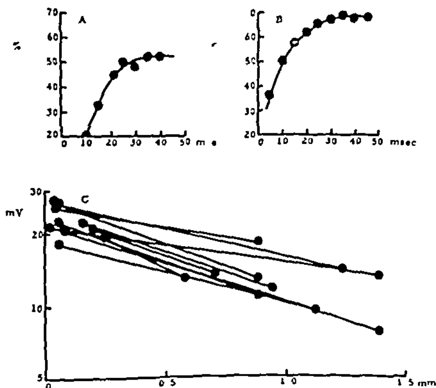


Fig 22 Spread of subthreshold depolarization along muscle fiber *A* corresponding to the experiment Fig 21 shows the subthreshold depolarization at electrode V_2 (*ordinate*) in per cent of that at V plotted at different times (*abscissa*) from onset of linearly rising stimulus *B* similar determinations on another fiber V 80μ and V 1380μ from stimulating electrode *C* simultaneous membrane depolarizations (*ordinate* logarithmic scale) about 5 msec prior to spike discharge at various distances from stimulating electrode (*abscissa*) the straight lines joining points representing the two values obtained from each individual fiber in 10 experiments

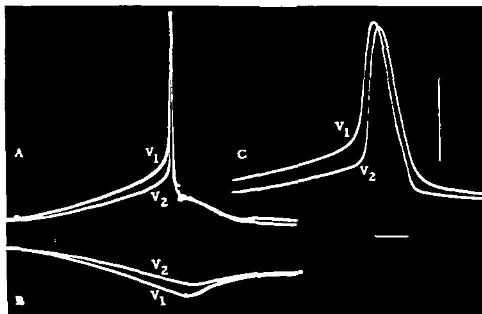


Fig 23 *A* depolarizations 80μ (electrode V_1) and 1380μ (V_2) from stimulating electrode *B* hyperpolarizations at same electrode positions during reversed stimulus of same gradient *C* final phases of recordings in *A* at faster sweep speed Horizontal bar 20 msec in *A*—*B* 2 msec in *C* Vertical bar 50 mV

fiber The results indicate that the subthreshold depolarization in response to a linearly rising current is largest in the immediate vicinity of the stimulating electrode

This does not exclude the possibility that the activation processes in the membrane, *viz* the generation of the active subthreshold potential, may occur in specific areas of the fibers and spread from these areas to the site where they are recorded In order to get some idea of the spatial origin of the active component of the subthreshold depolarization the magnitude of this component was determined at various points of the fiber by means of the experimental procedure illustrated in Fig 23 In this figure record *A* shows depolarizations at distances of 80μ (electrode V_1) and 1380μ (V_2) from the stimulating electrode while record *B* shows hyperpolarizations at the same electrode positions in response to a reversed linearly rising stimulus of the same gradient If the hyperpolarization is subtracted from the depolarization (cf section *III*) a considerably larger difference will result at V_1 than at V_2 In twelve fibers tested at various distances between the stimulating and the two recording electrodes this difference, which may be taken as an approximate measure of the magnitude of the active component of the

membrane potential changes, was regularly found to be most pronounced at the electrode next to the point of stimulation. The active component of a subthreshold depolarization should thus be largest in the area adjacent to the stimulating electrode. From the present observations it cannot be decided whether this is due to active subthreshold potentials being generated only within a limited region of the fiber membrane and spreading electrotonically from this area, or whether it is due to an ever larger portion of the fiber membrane being activated as the polarization spreads along a fiber. In analogy with events in nerve (cf. Pumphrey, Schmitt and Young 1940), it may however be assumed that each part of a muscle fiber membrane being depolarized to a certain extent gives rise to subthreshold activation processes.

The fact that both subthreshold depolarization and active subthreshold depolarization are largest in the vicinity of the stimulating electrode suggests that the active subthreshold responses are not generated in any specific areas of the fiber membrane, hence, in end plate free parts of the fiber the membrane may be presumed to have the same excitability properties in different regions, and the position of the stimulating electrode can thus be regarded as the factor that decides where the activation processes are initiated and where they attain their highest intensity.

Spike responses. Previous investigations using electrical shock stimulation of muscle have shown that the point of origin of an impulse is frequently located at some distance from the stimulating electrode (Buchthal and Sten Knudsen 1959, Werman, McCann and Grundfest 1961). The site of spike initiation during a stimulating current of the type used in this investigation was determined by calculations of the latencies of the spike responses during simultaneous recordings at two different points of the membrane at varying distances from the point of stimulation. This is illustrated in Fig. 21 *D* showing membrane potential changes 160 and 940μ from the stimulating electrode on a faster time scale than in *C*, so that only the final phases of the stimulation were included in the record. The spike response can be seen to occur earlier at V_1 than at V_2. If the propagation velocity is presumed to be constant, the result obtained thus implies *either* that a spike started at the stimulating electrode, *or* that a spike was initiated somewhere between V_1 and V_2, closer to V_1 than V_2, and that an impulse was then propagated in both directions. This latter alternative can however be ruled out, since the spike will first reach V_1 even if V_2 is moved closer to V_1, the practical limit being 30μ. This strongly supports the assumption that during a linearly rising stimulating current the propagated response is set up in the immediate vicinity of the stimulating electrode. This assumption gains further support from some observations made in connection with membrane potential

changes recorded at either side of, and at equal distances from the stimulating electrode. This is illustrated in Fig 24, showing spike responses obtained 900μ from the stimulating electrode From the superimposed recordings of responses obtained at either side of the stimulating electrode it may be seen that the spikes occur simultaneously This is actually what might be anticipated if the spike is initiated at the stimulating electrode and travels at the same velocity in both directions.

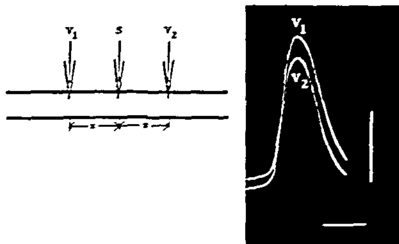


Fig 24 Spread of spike responses along muscle fiber Recordings at either side of and at equal distances x = 900μ from stimulating electrode electrode arrangement shown in diagram to left of record S stimulating electrode V and V recording electrodes. Different base lines to facilitate distinction between the two responses Horizontal bar 2 msec. Vertical bar 50 mV

Assuming that the spike starts at the stimulating electrode the conduction velocity in individual muscle fibers can be determined by calculating the latencies between the spike potentials obtained in an experiment of the type illustrated in Fig 21 *D* The mean value obtained from calculations on eleven fibers in three different muscles at a temperature of 14°C was 1 8 m/sec, with a range of variations from 1 2 to 2 3 m/sec these results agree reasonably well with previous determinations of the conduction velocity in individual frog muscle fibers Hakansson 1956 Tasaki and Hagiwara 1957, Buchthal and Sten-Knudsen 1959,

In fibers which for some reason exhibited a low resting potential around the stimulating electrode only small spike responses were obtained in those

regions, these spikes were graded, i.e., they changed with the stimulus gradient (Knutsson and Skoglund 1963). These responses may sometimes be built up to spikes of normal amplitude as the response is propagated along the fiber (Fig. 25 A), but in other cases only electrotonic spread in the area next to the stimulating electrode occurs (Fig. 25 *B*), while a propagated spike develops only at some distance from the stimulating electrode (Fig. 25 *C*).

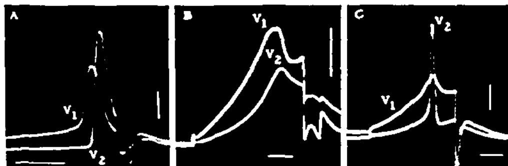


Fig. 25. Different types of responses to linearly rising stimulus currents in slightly depolarized muscle fibers. Electrode arrangement as in Fig. 22. *A*, spike successively built up during propagation: at V_1 low amplitude spike 10[illegible]0μ from stimulus site (S); at V_2 spike of normal amplitude 2[illegible]30μ from S. *B*, only electrotonic spread of small "spike": V_1 150μ and V_2 830μ from S. *C*, propagated spike develops at a distance from stimulating electrode: V_1 630μ and V_2 2080μ from S.
All vertical bars 20 mV. All horizontal bars 10 msec.

These types of propagation were observed only in fibers exhibiting low resting potential around the stimulating electrode. A possible explanation may be a local decline in excitability in these cases, the primary cause of which may have been a prolonged depolarization of the membrane within a limited region of the fiber, but other factors, such as damage inflicted to the membrane, cannot be ruled out. It can be safely concluded, however, that these types of spread have not occurred in the experiments described in sections I and III above (except the depolarization experiment Fig. 19) since the very characteristic spike changes connected with them, i.e., diminishing amplitude and prolonged spike duration, could not have escaped notice.

brane and is thus not at variance with previous determinations of the AC impedance in muscle (Dubuisson 1935, Katz 1942) A decrease in conductance during hyperpolarization, as indicated by these impedance measurements, does in itself give rise to an increased *RI* drop This may be assumed to be associated with a decrease of the membrane permeability to ions and if for instance the permeability to potassium ions is decreased one may expect a change in the membrane potassium equilibrium potential resulting in a shift of the membrane potential toward zero level (cf Grundfest 1962 Baker Hodgkin and Shaw 1962) Two separate processes, one leading to an increase, and the other to a decrease, of the membrane potential change, may then act in opposite directions, and only when one of them predominates would the result differ from a passive charge.

Interpretation of subthreshold depolarizations In their studies on frog muscle fiber Kuffler (1942) and Katz (1942, 1948) have shown that the voltage changes occurring at the cathode and the anode are of equal amplitude when the stimuli applied are less than about one third of threshold strength whereas with stronger subthreshold stimuli the cathodal response is larger than the anodal. Analogous results were obtained in investigations using square wave stimulation and intracellular recording (Fatt and Katz 1951) There is thus satisfactory qualitative agreement between these previous findings and the results obtained in the present study using linearly rising currents and the present results also agree well with those reported by Jenerick (1959) in his analysis of subthreshold depolarizations during square wave stimulation. However variations in the rate of the depolarization have not previously been systematically studied and the present work thus provides additional information about the subthreshold membrane depolarizations in muscle fiber

Section II above describes how the passive processes may change with variations of the current gradient, while section III is concerned with changes in the active processes with the rate of rise of the current. In conformity with previous interpretations of subthreshold depolarizations the view is advanced that a depolarization in response to a linearly rising current is in part a result of a passive discharge of the membrane capacitance and in part a result of active processes in the membrane proper such as conductance changes and/or current generation these latter processes resulting in a further discharge of the polarized membrane This concept of an interaction of active and passive processes may provide a fairly adequate explanation for the subthreshold depolarizations observed on varying the stimulus gradients.

Properties of the active subthreshold potential The active subthreshold potential in muscle fiber was found to be a *graded* response both in regard

to rate of rise and amplitude Its rate of rise was thus shown to vary considerably, and in a characteristic manner, with the current gradient, and its amplitude showed a continuous and approximately exponential increase with time during depolarization it was abolished if the current was switched off before the critical membrane potential was attained, otherwise it turned into a spike response A close analysis revealed that the amplitude of ASP could be approximately expressed as a function of dI/dt and time

As appears from the results described in section III, a larger depolarization is necessary for the onset of ASP the faster the rise of the stimulating current Whether this actually implies that the activation processes in the membrane are initiated at different depolarization levels is difficult to say, since the onset of ASP was determined only when there was a measurable difference between depolarization and hyperpolarization What can be established by the determinations is consequently, only when the active subthreshold potential has reached a certain magnitude and by then the activation processes have presumably already started Since the rate of rise of ASP varies with the stimulus gradient, calculations of the actual depolarization required to start activation processes would be hazardous and it cannot be excluded that rather small depolarizations give rise to changes in the properties of the muscle fiber membrane, as suggested for nerve fiber (Lorente de No 1947) this concept is also supported by observations of the continuous AC impedance changes during depolarization (Dubuisson 1935, Katz 1942) The fact that ASP becomes apparent at different levels of depolarization at different rates of depolarization indicates however that the changes of the membrane properties are time dependent

How far the activation processes may spread along a fiber membrane during a subthreshold depolarization could not be established by the present investigation, but the active subthreshold potential could be shown to reach its highest amplitude at the point of initiation of the propagated response, *viz* in the immediate vicinity of the stimulating electrode and it could also be shown that there was in this region a gradual transition from subthreshold depolarization to propagated response

Interdependence of ASP and threshold of stimulation The fact that ASP attains a larger maximal magnitude during a slow than during a rapid depolarization seems to be causally related to the changes in stimulus threshold on variation of the stimulus gradient During a slowly rising current the active subthreshold potential constitutes a comparatively large part of the depolarization to critical membrane potential level, whereas during a rapidly rising current ASP is a relatively small fraction of this depolarization As dI/dt is reduced there is thus an increase of the depolarization induced by

activation processes in the membrane Consequently, a fall in stimulus threshold should be expected with diminishing stimulus gradient, and this postulate was actually verified by Knutsson and Skoglund (1963) A decrease in stimulus threshold with diminishing stimulus gradient, 'negative accommodation' (cf Skoglund 1945), is however also dependent upon the effect of the membrane time constant on polarization during a linearly rising current, *viz* a gradual reduction of the voltage current ratio as dI/dt is increased The changes in stimulus threshold on variation of the stimulus gradient can thus be presumed to be, in part, an effect of a change in magnitude of ASP and, in part, a time constant effect A fall in excitability during slowly linearly rising currents, accommodation (cf Skoglund 1942), could be observed only following certain changes in the experimental conditions, an account of these observations will be given elsewhere

Origin of the active subthreshold potential According to Adrian and Freygang (1962) there is a fall in conductance of the frog muscle fiber membrane during subthreshold depolarization, anomalous rectification This would increase the polarizing effect of the stimulating current and might thus explain why a depolarization becomes larger than a passive membrane *potential change* It should, however, be taken into consideration that this conductance fall was deduced from the fact that a polarization (V_b) relatively close to a stimulating electrode minus polarization (V_a) farther away from that electrode divided by the latter polarization, thus $(V_b—V_a)/V_a$, gives a value that is reduced as the depolarization increases It is obvious that such a change need not imply a conductance fall across the membrane but may also be due to, *e g* currents generated within the membrane during depolarization (cf Hodgkin, Huxley and Katz 1949) A progressive rise in membrane conductance during cathodal subthreshold stimulation, as revealed by measurements of the AC impedance (Katz 1942) is however not in itself a sufficient explanation for the acceleration of the depolarization on subthreshold stimulation A rise in the membrane conductance during depolarization would rather be expected to result in a smaller depolarization than a passive membrane potential change To find a causal connection between the progressive rise in membrane conductance and the observed acceleration of the membrane depolarization, one would have to assume some process accompanying the conductance changes (Cole and Baker 1941, Katz 1947)

One process of this kind is an increase of the membrane permeability to sodium ions (Marmont 1949 Hodgkin and Huxley 1952 c) Such an increase is consistent with experimental observations on sodium currents across the membrane made in voltage clamp experiments on nerve (Hodg

kin and Huxley 1952 a Hodgkin 1957), it is also supported by the observations that the amplitude of the active subthreshold potential is reduced in nerve on lowering of the extracellular concentration of sodium ions (Wright 1956) and is abolished in muscle in the absence of extracellular sodium ions during square wave stimulation (Jenerick 1959) and also, as shown in the present investigations, during linearly rising currents This concept gains further support from the observation that 'sodium currents during depolarization are influenced in the same way as ' active subthreshold responses by changes in the calcium concentration (Arvanitaki 1939, Frankenhaeuser and Hodgkin 1957 Frankenhaeuser 1957, Jenerick 1959 Shanes *et al* 1959) and by local anesthetics (Taylor 1959 Inoue and Frank 1962) A selective change of the permeability to sodium ions may however, not be an absolute prerequisite for the appearance of active subthreshold responses (Teorell 1962) which can actually be thought to occur within a large number of quite different physiological systems (FitzHugh 1961)

If the active subthreshold potential is a consequence of currents generated in the membrane its rate of rise is obviously dependent not only on these currents but also on the membrane resistance and the membrane time constant As the resistance varies during a subthreshold depolarization the time constant is likely to be altered This implies a serious limitation of the possibilities to determine the magnitude of the activation currents from the voltage changes observed It should however be possible to make certain suggestions concerning the rate of rise of the activation current Since the time constant effect on the membrane potential changes is most pronounced during rapid polarizations and less marked during slow polarizations the large differences in the rates of rise of ASP at different stimulus gradients would obviously correspond to still larger differences in the rates of rise of the activation currents The membrane can consequently be supposed to be able to generate currents of varying rate of rise within very wide limits during subthreshold depolarizations whereas this ability is usually lost when the critical membrane potential level is attained

If these currents are assumed to be effects of increased membrane permeability to sodium ions there would be—as a consequence of the progressive increase of ASP—a progressive rise in the Na permeability during a subthreshold depolarization The velocity of the permeability change would also vary with the rate of rise of the stimulus From the results obtained in the present investigation it is not possible to estimate the permeability changes since ASP should not be proportional to the conductance changes, owing to the time constant of the membrane The very pronounced differences observed in the active subthreshold potential at one and the same membrane

potential level during depolarization of different rates of rise suggest, however, that the conductance changes are dependent both on membrane potential and time which is in good agreement with concepts previously advanced with respect to nerve (Hodgkin and Huxley 1952 b, Frankenhaeuser and Hodgkin 1957). Thus, the sodium conductance of the membrane during a subthreshold depolarization may rise with increasing depolarization but the changes in conductance may follow the membrane potential changes more readily, the slower the depolarization.

The mechanisms underlying the activation processes, above expressed in terms of changes in membrane conductance or membrane permeability, have been the subject of much controversy. Since ASP was abolished on removal of sodium ions from the extracellular fluid, it may be reasonable to assume that the sodium flux is altered during subthreshold depolarization, this would also be consistent with concepts previously advanced (Hodgkin 1957, Hodgkin and Horowicz 1959, Strickholm 1962). Nothing is however so far known about the nature of the transducer actions (Grundfest 1957) causing the changes in conductance of the cell membrane. One possible explanation which may account for at least part of the conductance increase may be water flow changes observed across negatively charged membranes (Teorell 1956, 1959 a, b). Since these changes are rather slow processes they might explain the marked time dependence of the subthreshold conductance changes. There may be other possible explanations, the present investigation supports theories implying voltage- and time dependent ion transports during activation, the velocity of which may vary within very wide limits during depolarizations to a critical transmembrane potential.

SUMMARY

1 The present investigation was undertaken to study the responses to linearly rising current in single frog muscle fibers by means of internal electrodes one for applying stimulus current across the fiber membrane and one or two for recording the concomitant membrane potential changes Depolarizations from resting potential to critical membrane potential were studied, as well as hyperpolarizations induced by stimuli of opposite direction and threshold strength

2 In order to estimate what part of a depolarization should be regarded as subthreshold the critical membrane potential was determined as the membrane potential at the largest depolarization not resulting in a spike when series of current pulses of successively larger amplitude were applied

3 When the stimulus gradient was altered the subthreshold depolarization rate changed the spike latency variations ranging from 5 to 1500 msec During rapid depolarizations there was a non linear increase of the subthreshold depolarization with time during very slow depolarizations on the other hand, only the final phase was non linear

4 The rate of the depolarizations was estimated by means of approximate graphic differentiation

(a) During rapid depolarizations attaining critical membrane potential in 5 to about 100 msec there was a marked increase in the depolarization rate throughout the subthreshold depolarization

(b) During depolarizations attaining critical membrane potential in about 100—500 msec there was only a slight increase of the depolarization rate in the first phase, and a marked increase in the final phase of the subthreshold depolarization

(c) During very slow depolarizations taking about 1 sec to reach critical membrane potential the rate was constant in the first phase of the depolarization whereas there was a marked increase in the depolarization rate in the final phase

5 Hyperpolarizations in response to stimulus current of threshold strength reached a maximum amplitude of 20—30 mV

potential level during depolarization of different rates of rise suggest, however, that the conductance changes are dependent both on membrane potential and time, which is in good agreement with concepts previously advanced with respect to nerve (Hodgkin and Huxley 1952 b, Frankenhaeuser and Hodgkin 1957) Thus the sodium conductance of the membrane during a subthreshold depolarization may rise with increasing depolarization, but the changes in conductance may follow the membrane potential changes more readily, the slower the depolarization

The mechanisms underlying the activation processes above expressed in terms of changes in membrane conductance or membrane permeability, have been the subject of much controversy Since ASP was abolished on removal of sodium ions from the extracellular fluid, it may be reasonable to assume that the sodium flux is altered during subthreshold depolarization, this would also be consistent with concepts previously advanced (Hodgkin 1957, Hodgkin and Horowicz 1959, Strickholm 1962) Nothing is however so far known about the nature of the transducer actions (Grundfest 1957) causing the changes in conductance of the cell membrane One possible explanation which may account for at least part of the conductance increase may be water *flow changes observed across negatively charged membranes (Teorell* 1956 1959 a, b) Since these changes are rather slow processes they might explain the marked time dependence of the subthreshold conductance changes There may be other possible explanations, the present investigation supports theories implying voltage and time dependent ion transports during activation the velocity of which may vary within very wide limits during depolarizations to a critical transmembrane potential

(c) The amplitude of ASP at one and the same membrane potential varied with the depolarization rate, the maximum value of ASP, varying between about 5 and 15 mV, increased as the current gradient was reduced

9 Presetting of the membrane potential of a fiber at various depolarized levels prior to onset of a linearly rising current pulse resulted in a change in the rate of rise of the active subthreshold potential, the more depolarized the fiber the faster was the increase of ASP

10 On substitution of the sodium ions in the extracellular fluid by choline ASP was abolished and rectification was observed at about 20—40 mV depolarization The slower the rise of the stimulus current the smaller was the depolarization at which rectification occurred, and the slower was the increase of the rectification

11 Subthreshold depolarizations during linearly rising current are largest in the area adjacent to the stimulating electrode and diminish in amplitude with the distance from the site of stimulation The reduction with distance varies during a just subthreshold depolarization but is about constant in its final phase The difference between a depolarization and a hyperpolarization in response to current pulses of equal strength was determined at various distances from the stimulating electrode and was found to be larger the shorter the distance from the site of stimulation which indicates that also the active response is largest close to the stimulating electrode

12 The propagated response was usually set up in the immediate vicinity of the site of stimulation but when the resting potential was slightly reduced it often started at some distance from the stimulating electrode

The propagation velocity in single frog muscle fibers was 1 8 m/sec range 1 2—2 3 m/sec, which is in agreement with previous determinations

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Contents

Chapter 1

General Introduction

The notion that the stretch reflex is an element in parkinsonian rigidity was first derived from the observation that rigidity is partly abolished after section of the dorsal roots (FOERSTER 1911, 1921; POLLOCK and DAVIS 1930).

At a time when only one kind of motoneurone was known, the conception of the stretch reflex mechanism implied only one factor determining the muscle response to a given stretch: the excitability of a uniform population of motoneurones. The use of the tendon jerk as an index of the number of motor units available for the stretch reflex in clinical practice and investigation was adequate for the contemporary physiological knowledge.

The discovery that the stretch sensitivity of the muscle spindles is regulated by the γ motoneurones introduced a second factor determining the reflex contraction to a given stimulus (LEKSELL 1945; GRANIT 1955). An adequate clinical analysis of stretch reflex pathophysiology presumes methods that indicate γ system activity or muscle spindle sensitivity in humans. One method of indicating the γ motoneurone control of motor phenomena is based upon the possibility of producing blocks selective to the γ fibres of a peripheral nerve (RUSHWORTH 1960). Another method rests on the assumption that the excitability of the muscle spindles under certain conditions is indicated by a comparison of the reflex response to tendon taps and to electrical stimulation of muscle spindle afferents (HASSLER 1956; STRUPPLER and FLEISCHHAUER 1955). However these two methods of studying the function of γ motoneurones and muscle spindles gave contradictory results when applied to parkinsonism. The first method indicated a high, the second a low fusimotor activity in parkinsonian rigidity.

The picture of the stretch reflex mechanism has been further complicated by the discovery of two types of α motoneurones, one type responding phasically and the other tonically to muscle stretch (GRANIT, HENATSCH and STEG 1956) and by the demonstration of a dual motor control of the muscle spindles (BOYD 1962; JANSEN and MATTHEWS 1962; MATTHEWS

1962) The methods applicable to humans do not permit isolation and control of all the interdependent factors that influence the muscle response to mechanical activation of muscle spindles Reliable information on the spinal and muscle spindle functions taking part in the stretch reflex mechanism is to day only available in animal experiments by direct recording from single efferent and afferent nerve fibres innervating the muscle and its receptors A method of inducing a state similar to parkinsonism in animals would provide conditions for an electrophysiological analysis of the stretch reflex mechanism in rigidity

Although a great body of experience has been collected on the effects of experimental brain lesions some of which imitate other extrapyramidal motor diseases no such way of producing a syndrome of akinesia rigidity and tremor simulating parkinsonism has yet been found (DENNY BROWN 1961 1962)

It has however been known for about a decade that reserpine and chlorpromazine as well as other phenothiazine derivatives when given in high doses have side effects resembling parkinsonism Although this state can be reproduced in animals the possibility of using it as a model syndrome for the neurophysiological analysis of parkinsonian rigidity has not been utilized

The final test of the theories on parkinsonian rigidity must await a development of the methods of clinical neurophysiology The analysis of the reserpine syndrome however, could be supposed to reveal mechanisms of rigidity and lead to the formulation of hypotheses on parkinsonian pathophysiology suggesting appropriate methods of clinical analysis

The difficulties of recording the activity of single peripheral nerve fibres in the usual preparations without abolishing the motor phenomenon to be studied led to an investigation of the innervation of the segmental tail muscles in the rat These muscles are small enough to permit exact recordings of single motor unit contractions The activity of single spontaneously active nerve fibres can be recorded and identified in the thin intact spinal roots and nerves innervating the muscles simultaneously with the electromyographic demonstration of rigidity and tremor

The next chapter of this study is a morphological description of the rat tail muscles and their innervation The third chapter is a presentation of the technique of preparation and recording used in the physiological experiments In the fourth chapter the function of the different types of motoneurones innervating the extrafusal and intrafusal muscle fibres is studied and discussed A description of the motor syndrome induced by reserpine is given in the fifth chapter The sixth chapter reports on the change of activity in efferent axons and motor units intro

duced simultaneously with rigidity after reserpine injections. The seventh chapter is a general discussion. It commences with a presentation of neuropharmacological connections between the parkinsonian and reserpine syndromes. The data from clinical muscle spindle and stretch reflex studies in parkinsonian patients are discussed on the basis of the mechanisms revealed in the electrophysiological analysis of reserpine rigidity.

Chapter II

Anatomy

A study of the anatomy of the segmental tail muscle innervation in the cat (v. SCHUMACHER 1909) discloses an organization of 23 muscular segments innervated from the third sacral and the six spinal coccygeal segments. On each side of the tail there are two longitudinal nerve trunks, one dorsal and one ventral. Each segment contains three pairs of muscles, one dorsal pair receiving innervation from branches of the dorsal nerve trunks, one lateral pair and one ventral pair both innervated from the ventral nerve trunks.

A morphological description of the rat tail muscles

The morphological organization of the segmental muscles and their innervation in the rat tail is essentially the same as in the cat.

The rat tail contains 27 vertebrae. In rats of 350—400 g size the mean length of the vertebrae is 1 cm. The most proximal as well as the most distal are somewhat shorter than the middle segments. Three pairs of long tendons emanating from muscles at the base of the tail and three pairs of short tendons of muscles emerging from the next proximal segment insert into the proximal end of each vertebra. On each side of a vertebra there is one dorsal, one lateral and one ventral segmental muscle having a length of 10 mm and a weight of about 10 mg.

The rat tail is innervated from six pairs of dorsal and ventral coccygeal roots. One spinal segment usually innervates four muscular segments. Outside the vertebral column the roots join to form one dorsal and one ventral longitudinal nerve trunk on each side. The dorsal muscles and the skin at the dorsal aspect of the tail are innervated from the two dorsal nerve trunks. The lateral and ventral muscles and skin regions get their innervation from branches of the ventral longitudinal nerve trunks. The nerve branches innervating the lateral muscles are very long and thin muscle nerves containing no skin afferents. The nerves to the dorsal and ventral muscles are mixed and contain skin nerve fibres branching off from the muscle nerves very close to the muscles.

An histological examination of serial hematoxylin van Gieson stained cross sections of the lateral segmental tail muscles was made to study the occurrence of muscle spindles. The muscles of the 10th to 18th segments, the ones most often studied, usually contain three muscle spindles each. In the proximal part of the tail up to five spindles have been found. The most distal segments have muscles containing only one muscle spindle.

The spindles of the lateral segmental tail muscles contain both nuclear bag and nuclear chain intrafusal muscle fibres ([illegible] 1963). No Golgi tendon organs were found in the muscles ([illegible] 1963).

The muscle bundle at the base of the tail that through its long tendon acts on a vertebra is innervated from the same spinal segment as the segmental muscle inserting into the vertebra.

Summary

The lateral segmental tail muscles in the rat contain 1—5 muscle spindles and no Golgi tendon organs. They are innervated by long and thin muscle nerves not containing skin afferents.

Chapter III

Methods

In the physiological analysis of the efferent innervation of the segmental tail muscles 96 rats were used The neurograms of the tail muscle nerves were recorded on stimulation of coccygeal ventral and dorsal roots The response of muscle receptor afferents to muscle stretch and contraction was studied in filaments of the dorsal roots The mechanomyograms of the muscles were recorded on stimulation of ventral root filaments

The effects of reserpine on the activity of single efferent axons and motor units and on the spinal reflexes were investigated in 22 rats

Anaesthesia

Albino rats weighing between 350 g and 400 g were used In the study of the efferent tail muscle innervation the anaesthetic Nembutal was used 50 mg/kg injected intraperitoneally

The activity of efferent axons and motor units was recorded in intact rats after recovery from the tail operation Two per cent Fluothane added to a nitrous oxide oxygen mixture by a Fluotec evaporator gave good anaesthesia during the operation and the animal recovered completely 15 minutes after the end of the anaesthesia

The preparation of the spinal reflex experiments was made under anaesthesia with nitrous oxide and 50 mg/kg Viadril (Pfizer) injected intravenously in two doses through a fine plastic tube inserted into the tail vein of the right side After the operation a light anasthesia was continued with the nitrous oxide oxygen mixture and the remaining effect of Viadril

Immobilization

The laminectomized animals lay prone on a plate and were immobilized by clamps on wrists and ankles The vertebral column was stretched between one pin through the iliac crests and one under the spinal ligament connecting the Th 12 and L 1 spinal processes Two pins

penetrating the tissues close to the ventral aspect of the vertebral column gave a firm support to the lumbar vertebrae. In the spinal reflex experiments the head of the rat was enclosed in an anaesthesia mask made by a funnel and a rubber membrane.

The intact animals lay in a plastic cylinder and were fastened by atraumatic clamps fitting well around the ankles. The ankle clamps were fastened in the plastic cylinder that could be rotated around its axis. The arrangement made it possible to elicit vestibular reflexes by a partial rotation of the animal. The anaesthetic was administered through a funnel tightly fastened on the front end of the cylinder.

The tail was immobilized in a plastic (Vepesol) tray by a firm rigidly fastened pin penetrating an intervertebral disc.

Dissection

A longitudinal incision was made in the skin on the left side of the tail ventral to the longitudinal subcutaneous vein. The skin flaps were loosened from the subcutaneous tissues and fastened by pins pricked into the soft plastic material of the tray, taking all precautions to keep intact the circulation in the vein.

The long tendons from the basal tail muscles were removed to expose the lateral segmental muscles, the ventral nerve trunk and its branches between the 8th and the 24th segment. The tray was filled with liquid paraffin.

The muscle nerves were then microscopically dissected free to a length of about 10 mm without cutting the peripheral end of the nerve, thus leaving the muscle innervation intact.

Denervation was made by transection of both the nerve trunks on the right side, the dorsal left nerve trunk, the part of the ventral left nerve trunk lying distally to the freed muscle nerve, and all the nerve branches emerging from the nerve trunk proximally to the studied muscle nerve, thus leaving only this segmental tail muscle with intact innervation. In experiments in which the activity of single afferent fibres from the muscle was recorded in dorsal root filaments, the basal tail muscles of the left side also had to be denervated in order to reduce the number of active afferent fibres in the dorsal root.

Laminectomy was made by removal of the second to sixth lumbar vertebrae. A paraffin pool covered the exposed sacral and coccygeal spinal cord.

Temperature was measured by two thermometers, one in the dorsal and one in the tail paraffin pool, and controlled by an infrared heating

lamp directed at the animal. The temperature in the laminectomy pool was kept at 35—36°C and in the tail pool at 33—35°C.

Recording technique

Platinum electrodes were used. Two recording electrodes were attached to the nerve of a lateral segmental tail muscle. Bipolar recordings were also made from dorsal and ventral roots and root filaments. In some experiments two pairs of electrodes 2—10 mm apart were attached to muscle nerves or ventral roots. The arrangement with double pairs of recording electrodes made it possible to identify the active efferent and afferent fibres by their direction and their conduction velocity calculated from the conduction distance and the conduction time between the electrode pairs.

All these electrodes could easily be connected to the preamplifiers or stimulators. The Tektronix type 122 low level preamplifiers were coupled to the Tektronix dual beam 502 oscilloscope. A Grass kymograph camera was used for photographic recording.

The single action potential deflections recorded on the oscilloscope screen with the short interelectrode distance used had the shape of spikes of very short duration, which made the individual potentials of the neurogram more distinctly apparent. Each oscilloscope beam was connected to one electrode pair in the double pair arrangement for identification of the action potentials; the time lag between the deflections on the two oscilloscope beams indicated the conduction velocity of the action potential.

Two condensor discharge stimulators were used for electrical stimulation, which were isolated by General Radio isolating transformers type A1, giving a stimulus half decay time of about 40 usec.

Each stimulator had facilities for continuously variable delayed single stimuli and tetani with a continuously variable duration and a maximum frequency of 1000/sec.

The muscle tension was recorded by a semiconductor strain gauge myograph (Century of Tulsa Strainistor Mod. 711). A loading of the myograph by 1 g caused a myograph deflection of 18 µ.

The myograph was connected to the muscle by means of a hook inserted into the short distal tendon. The myograph was mounted on a hydraulic puller device designed for linear stretches of various speeds.

The electromyogram was recorded in the calf muscles with a concentric bipolar needle electrode.

Efferent Muscle Innervation

Introduction

Fast and slow striated muscles

RANVIER (1874) demonstrated slower twitch contractions and higher fusion frequencies in red muscles than in pale ones. DENNY-BROWN (1929b) stated that "fibres of similar speed of contraction form a group which is sharply delimited from other such groups and in most situations form a muscle head." Motor units of slow muscles discharge at slower rates and have lower thresholds of reflex activation than those of fast muscles (DENNY-BROWN 1929a). Small myographic deflections ascribed to single motor units discharging at slow rates on weak reflex activation were recorded in the tendons of m. soleus (ECCLES and SHERRINGTON 1930) and of m. tibialis anterior (GORDON and PHILLIPS 1953) in the cat. Similar deflections were observed in the human m. tibialis anterior (GORDON and HOLBOURN 1949). The contraction time corresponded to that of slow muscles. The analysis of single motor units in frog muscles showed one type with "all or none" twitches preceded by propagated action potentials and another type with graded contractions without propagated action potentials (TASAKI and MIZUTANI 1944, KUFFLER and GERARD 1947).

Muscle efferents

ECCLES and SHERRINGTON (1930) distinguished two calibre groups of efferent myelinated fibres in cross sections of ventral roots. O'LEARY, HEINBECKER and BISHOP (1935) found a small γ wave after the big α potential in the efferent neurogram. LEKSELL (1945) demonstrated that the γ fibres innervate the intrafusal muscle fibres of the muscle spindles and serve as regulators of sensory activity originating in the spindles.

GRANIT, HENATSCH and STEG (1956) differentiated two types of motoneurones, the one responding phasically, the other tonically to reflex activation. ECCLES, ECCLES and LUNDBERG (1958) found that fast muscles

are innervated from rapidly conducting axons of α cells with short after hyperpolarizations and slow muscles from slowly conducting neurones with long after hyperpolarization periods. These motoneurones were assumed to be identical with the phasic and tonic motoneurones described by GRANIT, HENATSCH and STEG.

Recently a dual motor innervation of the spindles from γ_1 and γ_2 motoneurones has been proposed (BOYD 1962, JANSEN and MATTHEWS 1962).

The γ loop

De-afferentation of a limb by section of the dorsal roots abolishes intercollicular decerebrate rigidity (SHERRINGTON 1898). This property of decerebrate rigidity is dependent on an intact proprioceptive pathway and got its interpretation when the hypothesis of the γ loop was introduced (GRANIT *et al* 1955, GRANIT 1955). The intrafusal muscle fibre contraction set up by the γ fibre activity excites the primary stretch receptor endings to an afferent discharge that through the stretch reflex arc elicits a muscle contraction. A transection of the dorsal roots will open the loop and eliminate the reflex contraction. The decerebrate rigidity being dependent on the intact γ loop could thus be described as a γ rigidity (GRANIT 1955).

Decerebration carried out by the anaemic method (POLLOCK and DAVIS 1931) by ligation of the basilar artery as well as the carotids induces a rigidity in the animal that differs from the intercollicularly decerebrated preparation. This rigidity is not eliminated by dorsal root section and is thus elicited by direct activation of the α motoneurones "by passing the γ mechanism". This form of decerebrate rigidity was described as an α rigidity (GRANIT 1955).

Results

Neurograms of the segmental tail muscle nerves

The neurograms of the muscle nerves to the lateral segmental tail muscles were recorded on stimulation of the ventral and dorsal coccygeal roots (Fig. 1). The efferent neurogram was composed of clearly distinguished single neurone action potentials which were divided into three usually well separated groups. The first group contained up to 8 single potentials with conduction velocities between 50 and 35 m/sec. The second group had maximally 3 potentials of velocities between 35 and 20 m/sec. In most neurograms the third group with conduction velocity

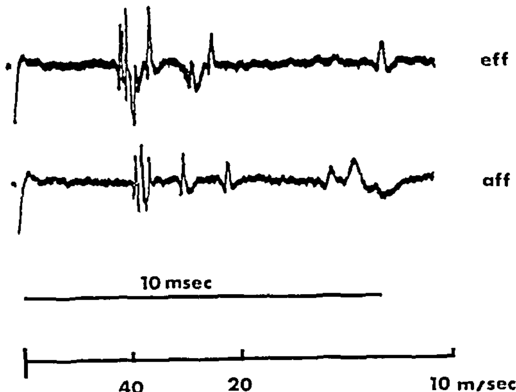


Fig 1 Neurograms recorded in a segmental tail muscle nerve on ventral root stimulation (*eff*) and dorsal root stimulation (*aff*) Three groups of potentials are seen in both efferent and afferent neurograms Conduction distance 133 mm Conduction velocities indicated below time Shock artifacts demonstrated

within the 20—10 m/sec range consisted of only one single action potential In some proximal muscle nerves two or occasionally three potentials were seen in the last group The efferent neurograms of the ventral and dorsal segmental tail muscles showed a similar composition

The tail muscles also received a few efferent fibres that had conduction velocities of 0 5—1 m/sec These unmyelinated fibres were firing spontaneously in bursts synchronous with breathing and could be activated by stimulation of the sympathetic trunk (Fig 2)

The afferent neurograms recorded in the muscle nerve on stimulation of the whole dorsal root were usually not so clearly organized in well separated groups as the efferent neurograms In some neurograms however the separation into three groups was evident The number of single potentials in each of the two first groups did not exceed 5 The third group which was not represented in all afferent neurograms had 1 2 or 3 potentials The conduction velocities of the first two groups fell within

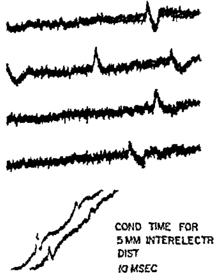


Fig 2 Spontaneously discharging efferent fibres (that can be activated on stimulation of the sympathetic trunk) recorded in a segmental tail muscle nerve Conduction velocity is calculated from the conduction time between two pairs of recording electrodes 5 mm apart It is 0 8 m/sec for one and 0 5 m/sec for another fibre

the range of 40—20 m/sec without any distinct demarcation between the groups The third group of potentials had conduction velocities between 20—10 m/sec Usually the fastest afferent single action potential had a conduction velocity slightly lower than that of the fastest efferent one

Functions of muscle afferents

The responses to muscle stretch and contraction were studied in isolated muscle afferents in fine dorsal root filaments The afferent fibres of the first two groups paused during extrafusal muscle twitches (Fig 4) No afferents responded with the increased discharge frequency during muscle twitch which is characteristic of Golgi tendon organs

The stretch responses of the fastest afferent fibres had an initial dynamic phase corresponding to a linear extension of the muscle followed by a static phase during maintained stretch (Fig 4) The slower afferents within the 40—20 m/sec range responded statically without the initial dynamic burst There was usually no distinct demarcation in conduction velocity between the first two groups and it was thus difficult to determine if all of the first and none of the second had this dynamic response

The first group of afferents corresponds functionally to the group Ia and the second to group II in the cat (cf LLOYD 1943 COOPER 1961) This terminology is applicable to the rat tail although its nerve fibres have lower conduction velocities The absence of fibres showing Ia responses corresponds to the absence of tendon organs in the segmental tail muscles (IP 1963) The third group of afferent fibres was not studied

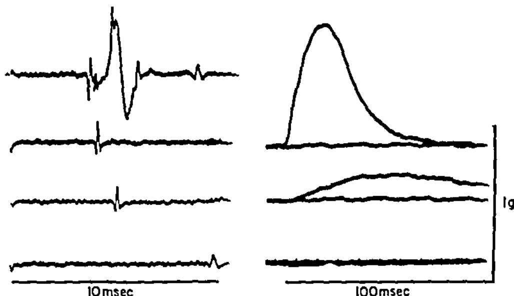


Fig. 3. *Left upper trace.* The neurogram recorded in a segmental tail muscle nerve on stimulation of a whole ventral root. The slow high amplitude deflection is the electromyographic artifact. The *lower left traces* demonstrate from above downwards the action potentials of one fast α, one slow α and one γ fibre stimulated in fine filaments of the ventral root. The filaments are stimulated more proximally than the whole root which is reflected in longer conduction times. *Right.* Isometric muscle contractions recorded by a strain gauge myograph attached to the tendon elicited on stimulation of a fast α fibre (*upper*) and a slow α fibre (*middle*). Stimulation of the γ efferent does not give any measurable contraction (*lower*).

As a general rule the number of potentials in the muscle nerve neurogram decreased from the proximal to the distal end of the tail. In the most distal segments afferent neurograms with only one group I and one group II potential could be found corresponding to the finding of one single muscle spindle in the muscle.

Functions of muscle efferents

The short tendon at the distal end of a lateral segmental tail muscle was cut close to its insertion and connected to the hook of the strain gauge myograph. A ventral root was divided dichotomically, the filaments were stimulated and the action potential recorded in the muscle nerve. The root was divided until filaments were found so fine that only one single fibre action potential remained in the neurogram. The action potential was identified by its place in the complete efferent neurogram recorded in the muscle nerve on stimulation of the whole ventral root

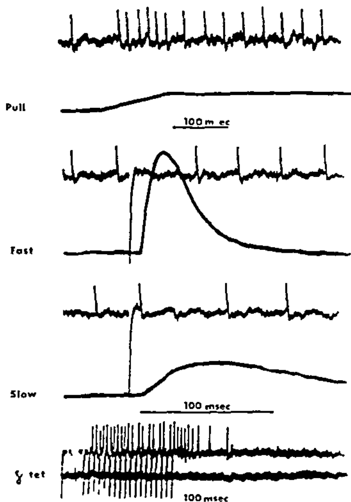


Fig 4 Response of a Ia afferent to muscle stretch and stimulation of single efferent fibres Upper beam in each pair demonstrates the activity of a Ia afferent in a fine dorsal root filament Lower beam shows extension in length at muscle pull and myographically recorded tension respectively The Ia afferent responds to *pull* with an initial dynamic and a later static discharge It pauses during both *fast* and *slow* motor unit contractions A tetanic stimulation of the γ efferent (*γ tet*) gives rise to a repetitive discharge in the afferent but not to muscle contraction Time for middle two pairs of traces the same Shock artifacts seen as downward deflections

When a motor axon of the fastest potential group was stimulated a muscle twitch of 18 msec contraction time was elicited The concentration produced by stimulation of a single motor fibre of the intermediate group had a contraction time of 50 msec The single slow fibre of the third group in the efferent neurogram did not give rise to any measurable tension (Fig 3)

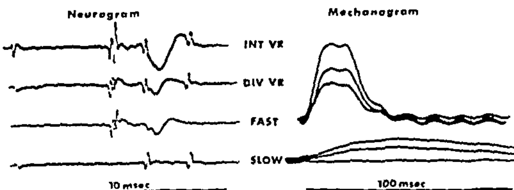


Fig 5 The left traces show the neurograms the right the myograms of the same tail muscle On stimulation of an intact ventral root four fast and two slow α fibres were recorded in the tail muscle nerve (*INT VR*) On division of the root into two filaments one fibre was lost this was demonstrated when the two filaments were stimulated together (*DIV VR*) In one of these two filaments three fast α fibres were present (*FAST*) in the other filament the two slow α fibres (*SLOW*) The superimposed mechanograms of one and two slow motor units and one two and three fast motor units are demonstrated They were obtained by progressively increased stimulus strengths for the respective ventral root filaments Mechanical oscillations in the myograph gave the wave artifact at fast contraction

It was a consistent finding in all experiments that fast α motor axons innervate rapidly contracting muscle fibres and slow α axons project to slowly contracting muscle fibres

A group I afferent fibre from the muscle studied was isolated in a fine dorsal root filament and identified as a I a fibre by its response to muscle pull and contraction The discharge of the I a afferent was recorded during stimulation of single efferent axons in each of the three groups There was a pause in the afferent discharge during the slow as well as the fast single contraction The afferent responded with a repetitive discharge during tetanic stimulation of the single fibre (Fig 4) of the last group in the neurogram This was thus identified as a γ fibre

Like the afferent fibres the efferent ones have considerably lower conduction velocities in the rat tail than in the hindlimb of the cat

Fast and slow motor units in the muscle twitch

In one experiment a ventral root containing fibres to a muscle nerve four belonging to the first and two to the second group of the neurogram was divided into two parts Three fast α fibres fell into the one and two slow α fibres into the other filament One fast α fibre was lost on

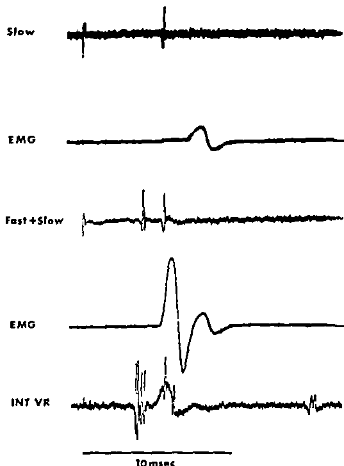


Fig 6 The lower trace demonstrates the neurogram recorded in the muscle nerve on stimulation of the intact ventral root (*INT VR*) After division of the root a filament containing one fast and one slow α efferent to this muscle is isolated This filament is stimulated and the nerve action potentials (*Fast+Slow*) as well as the electromyogram (*lower EMG*) are recorded After further division of this filament a slow α fibre to the muscle is stimulated separately The neurogram (*Slow*) and electromyogram (*upper EMG*) are shown The muscle action potentials are recorded with two electrodes on the muscle surface Both fast and slow motor units have biphasic action potentials

division of the root Fig 5 shows the superimposed mechanograms on stimulation of one two and three fast α fibres and of one and two slow α fibres All fast α fibres give rise to identical fast muscle contractions and both slow α fibres to identical slow contractions

Practically all motor units studied had contraction times either between 10 and 20 msec or between 40 and 50 msec Motor units contracting at intermediate speed were found only exceptionally

The peak tensions of the fast motor units were always higher than

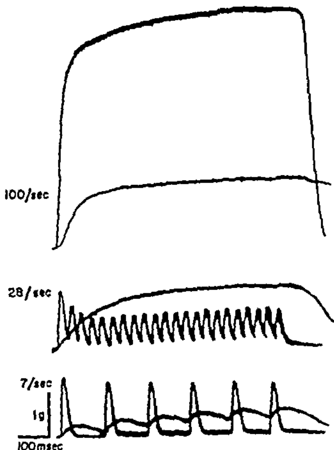


Fig 7 The mechanograms of a fast and a slow motor unit of the same muscle on repetitive stimulation of two ventral root filaments at different frequencies At a rate of 7/sec the slow unit contractions begin to fuse while no fusion occurs in the fast motor unit At 28/sec a smooth fusion is present in the slow unit contraction whereas the fast unit shows hardly any fusion A smooth contraction curve appears first at a rate of 100/sec for the fast unit

those of the slow motor units of a muscle the ratio varying between 1.5:1 and 5:1 Usually the tension relation between one fast and one slow motor unit studied in the same muscle was 2:1—4:1

The contraction time and peak tension are not substantially changed when the contraction of the slow motor units is superimposed on the twitch of the fast motor units of a segmental tail muscle

Propagated action potentials

One pair of electrodes with an interelectrode distance of 2 mm was attached to the surface of the muscle for recording the muscle action

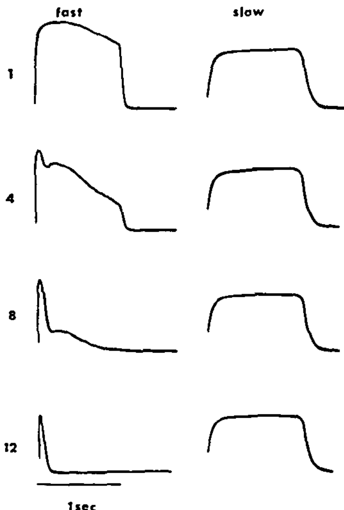


Fig 8 The resistance to fatigue of a fast and a slow motor unit in the same muscle A fast α fibre isolated in one ventral root filament and a slow α fibre in another filament were stimulated and the mechanograms of the fast and slow motor units were recorded isometrically Tetani of 100/sec frequency and 1 second duration were repeated at intervals of 2 seconds The first fourth eighth and twelfth tetanic contractions of the *fast* and the *slow* motor unit are demonstrated The slow motor unit contraction has the same shape after twelve successive tetani The fast motor unit contraction rapidly changes during repeated tetani indicating a low resistance to fatigue

potentials during stimulation of the fast and slow motor unit Fig 6 shows the electromyogram of a fast and a slow motor unit The biphasic configuration of the electromyographic potentials of slow as well as fast motor units indicates similar propagated action potentials The duration of the muscle action potentials is the same The amplitude of the slow

motor unit potential is smaller than that of the fast. The lower electromyographic potential amplitude of the slow motor units is a regular finding with this recording technique.

Fusion frequencies

Muscle contractions during repetitive stimulation at different rates of single fast and slow α fibres in fine ventral root filaments are demonstrated in Fig. 7. At a stimulation frequency of 7/sec the fast muscle contractions do not fuse but a beginning fusion of the slow contractions is noted. At a stimulation rate of 28/sec the slow contractions show maximal fused contraction and some fusion appears in the mechanogram of the fast muscle contractions. At a tetanic stimulation of 100/sec no further tension was added in the mechanogram of the slow muscle fused contraction but now a maximal fused contraction of the fast motor unit was produced.

Fatigue

The contractions were recorded of one fast and one slow motor unit stimulated tetanically at 100/sec in periods of 1 sec repeated at intervals of 2 sec. The fast motor unit contraction shows a considerable fatigue already after a few tetani and responds only with an initial twitch after 12 tetani. The slow motor unit contraction does not show any significant fatigue after 12 tetani (Fig. 8).

Discussion

Efferent innervation of muscle spindles

The finding of a single γ fibre innervating the muscle spindles of a tail muscle (STEG 1962 a, b) excludes a dual γ innervation of these spindles. A dual motor control of these spindles which have both nuclear bag and nuclear chain intrafusals presumes innervation from α efferents. The physiological evidence of efferent spindle innervation from α fibres (GRANIT, POMPEIANO and WALTMAN 1959 a, b) has been extended by LAPORTE and his co-workers. Their findings indicate motor innervation of spindles from branches of slow α axons increasing the dynamic sensitivity of the primary endings whereas the γ efferents mainly increase the static sensitivity (BESSOU, EMONET-DENAND and LAPORTE 1964).

The segmental rat tail muscles which are innervated by only one

, efferent and distally contain only one spindle present a unique preparation for muscle spindle studies

Motor innervation of extrafusal muscle

The small size of the segmental tail muscles and the small number of motor units they contain permit a direct and exact investigation of the contraction characteristics of all single motor units of a muscle The mechanomyograms recorded from the individual motor units of a muscle fall into two separate groups Fast contractions are elicited on stimulation of fast α fibres and slow contractions on stimulation of slow α fibres (STEG 1962 b) In contrast to the slow motor units in amphibians the slow mammalian motor units have propagated action potentials

Thus the old distinction of fast and slow types of striated muscle is distinctly demonstrated on the motor unit level in mammals

The distribution of fast and slow motor units in muscle

The generally accepted picture of muscle organization originating from the works of RANVIER and DENNY BROWN implies a clearcut separation of fast and slow elements most pronounced in muscles separated into heads Further detailed studies have confirmed that there are muscles having only slow motor units GORDON and PHILLIPS (1953) stated that "there is definitely no anatomical line of distinction between the rapid and slow elements" in the m tibialis anterior of the cat The slow caput mediale of m triceps brachii contains a fast element giving a rapid twitch on sub maximal nerve stimulation (ECCLES ECCLES and LUNDBERG 1958) The recording of single motor unit contractions in the rat tail muscles has demonstrated that they are mixed muscles BESSOU *et al* (1963) found a similar organization of the cat lumbricals

The myographic study of the segmental tail muscles also made evident that the contraction time and peak tension are not substantially influenced when the contraction of a minority of slow motor units is superimposed on the twitch of a majority of fast motor units A small but physiologically significant population of slow motor units in a predominantly rapid muscle may easily be overlooked in the twitch contraction curve of a muscle It can thus be stated that there is now increasing evidence from axial limb and digital muscles that fast and slow motor units are often mixed in mammalian striated muscle

Tetanic contraction and fatigue

Tetanically stimulated slow motor units have low fusion frequency and high resistance to fatigue compared with the fast units. This high resistance to fatigue of slow motor units corresponds well to a tonic discharge pattern at reflex activation (GRANIT, HENATSCH and STEG 1956). Motor units that are naturally only activated in short phasic bursts do not need to be resistant to fatigue. DENNY-BROWN (1929 a) ascribed the resistance to fatigue characterizing the postural stretch reflex to low frequency activation of motor units of slowly contracting muscles.

The slow motor units analysed in this study are likely to be tonically active in the maintenance of posture. Phasic movements are assumed to require the participation of the strong fast motor units.

Summary

The segmental tail muscle nerves contain a small number of fast and slow α efferents and usually only one γ efferent. Groups Ia, II and III afferent fibres have been identified but no Ib afferents. The fast α efferents innervate rapidly contracting extrafusal muscle fibres and the slow α fibres supply slow muscle fibres. The muscle fibres of both fast and slow motor units have propagated action potentials. The fast motor units have high fusion frequency and show fatigue rapidly. The slow motor units have low fusion frequency and are resistant to fatigue.

Chapter V

A model syndrome simulating parkinsonism

Introduction

If model syndromes simulating clinical disorders of posture and movement can be induced in animals electrophysiological investigation of the motor disturbances is possible although they cannot yet be analysed in humans The model syndrome cannot be regarded as identical with the clinical disorder but as a tool of research Its usefulness increases the closer the animal model syndrome approximates in motor phenomena and physiological observations to the human motor syndrome

A major method in the investigation of mechanisms controlling motor action is the analysis of effects on posture and movements of lesions and stimulation in the central nervous system A variety of motor phenomena analogous to neurological symptoms of extrapyramidal diseases have been produced with these techniques but a complete and characteristic parkinsonian syndrome of akinesia rigidity and tremor has not been reproduced (DENNY BROWN 1961 1962) Extrapyramidal motor symptoms resembling parkinsonism have been reported clinically and reproduced in animals after intoxication with carbon monoxide (DENNY BROWN 1960 1962) and manganese (VAN BOGAERTH and DALLEMAGNE 1945 COTZIAS 1958) The analogy of these motor phenomena to the clinical syndrome is not clearly demonstrated Some experimental states are not invariably reproducible

EVERETT *et al* (1956) claimed that tremorine produced an extrapyramidal motor syndrome with tremor resembling the parkinsonian but TRAUTNER and GERSHON (1959) found important differences and doubted the relation between the tremorine effects and parkinsonism

In 1952 DELAY and DENIKER introduced the recently synthesized phenothiazine derivative chlorpromazine into clinical use demonstrating its sedative property and ability to reduce motor hyperactivity Reserpine an alkaloid isolated from Rauwolfia serpentina was demonstrated to have largely the same clinical actions as chlorpromazine (WEBER 1954)

The term "neuroleptics" was introduced by DELAY and DENIKER (1955

1957) to denote a group of drugs the prototypes of which are chlorpromazine and reserpine. The criteria characterizing all neuroleptic drugs in spite of chemical differences and distinguishing them from other drugs of sedative or tranquillizing action are: 1. Strong sedation without actual narcotic action. The drugs are effective in states of excitation and agitation in patients and animals which are not controlled by classical sedatives. 2. Effects on autonomic nervous function. 3. Extrapyramidal symptoms (DELAY and DENIKER 1957). Within this general outline there are both quantitative and qualitative differences in action between the drugs.

The early reports on the extrapyramidal side effects of neuroleptic therapy presented a variety of hypo- and hyperkinetic symptoms. The symptomatology was interpreted by some authors as clearly parkinsonian, by others as pseudo-parkinsonian and some saw in it a combination of extrapyramidal and catatonic phenomena (DELAY *et al.* 1957). A basis for a comprehensive interpretation was provided by STECK's (1956) observation of the similarity in symptomatology to encephalitis lethargica and its sequelae. The comparative study was carried further by DELAY and DENIKER (DELAY and DENIKER 1957, 1961; DELAY *et al.* 1957; DENIKER 1960). "All revertible manifestations of a psychomotor, neurologic and vegetative order produced by different neuroleptics have already been classically described in Economo's encephalitis. However, neuroleptics do not produce all the disorders found in encephalitis" (DELAY and DENIKER 1961).

Segmental dystonia exemplified by oculogyric crises, torticollis, lordosis and scoliosis, which is characteristic of postencephalitic parkinsonism (DENNY-BROWN 1962) is also found in patients treated with neuroleptics (DELAY and DENIKER 1957, 1961). Akathisia, a motor restlessness, is one of the hyperkinetic phenomena found in the prodromic period to postencephalitic parkinsonism (SICARD 1923) and often accompanying parkinsonism. It is also seen during neuroleptic treatment (DELAY and DENIKER 1957, 1961). According to AYD (1961) the dystonic reactions to neuroleptic treatment are most common in childhood and youth, whereas akathisia has a prevalence for the middle age group.

The parkinsonian syndrome of akinesia, rigidity and tremor is the most common extrapyramidal side effect of neuroleptic therapy (DELAY and DENIKER 1957; GOLDMAN 1961). Its incidence is highest in old patients and AYD (1961) demonstrated that the age distribution corresponds exactly to that of naturally occurring parkinsonism. The akinesia includes a lack of kinetic initiative and of associated movements. The rigidity is often first manifested in an exaggeration of the biceps stretch reflex and then spreads in an asymmetrical manner to a general muscular rigidity

The tremor both of the postencephalitic and drug induced states varies in character between the physiologic shivering provoked by cold or emotion and the big amplitude slow oscillation typical of parkinsonism (MARIE and LEVY 1920 DELAY and DENIKER 1961)

A close connection between the extrapyramidal symptoms in humans and the state induced in animals on phenothiazine treatment was pointed out by COURVOISIER *et al* (1957) and on reserpine treatment by GLOW (1959) In the macacus rhesus monkey prochlorperazine treatment induced a fixed flexion posture and rigidity only influenced for short moments by strong activating stimuli A similar akinesia and rigidity was described in the dog after injection of the same phenothiazine and also tremor of the type found in parkinsonian patients (COURVOISIER *et al* 1957) An alternating 4—7/sec tremor with parkinsonian characteristics and distinguished from shivering was described in the reserpinized cat (STUART *et al* 1961) A syndrome with akinesia rigidity and tremor was induced in the rat by prochlorperazine (COURVOISIER *et al* 1957) and by reserpine (GLOW 1959) The rats always re established the upright posture when forced into adverse positions (COURVOISIER *et al* 1957) COLE and DEARNALEY (1960) found characteristic tail reflexes in the rigid reserpinized rat

Since the introduction of chlorpromazine a series of more potent phenothiazine neuroleptics have been synthesized producing comparable clinical effects at a lower dose Prochlorperazine could be rated 3—5 times more potent than chlorpromazine perphenazine 5—10 times and fluphenazine 10—20 times (AYD 1961) The fact that the extrapyramidal side effects as a rule are reversible (see however UHRBRAND and FAURBYE 1960) and have been claimed to be of a positive rather than negative significance for the therapeutic outcome (FLÜGEL 1956 BAYREUTHER 1958 HAASE 1961) has reduced the hesitance to intense neuroleptic treatment Later clinical reports have a higher incidence of extrapyramidal symptoms and typical parkinsonian syndromes than the early ones (DENIKER 1960 AYD 1961 FREYHAN 1961) The more potent neuroleptics give parkinsonian symptoms with a shorter latency than chlorpromazine (AYD 1961) In animals where there are no objections to intense neuroleptic treatment and the doses are usually much higher than in humans states with akinesia rigidity and tremor are produced with a very high incidence This state is invariably induced in rats 15—30 minutes after intravenous injection of 5—7 mg kg reserpine (GLOW 1959 STEG 1963 1964 ROOS and STEG 1964

Description of the reserpine syndrome in the rat

The pharmacological actions of reserpine are more suitable than those of other neuroleptic drugs for a comparison between the model syndrome and Parkinson's syndrome. Reserpine injection was thus used as the routine method to induce a state simulating parkinsonism.

Ten to fifteen minutes after an intravenous injection of 5—7 mg/kg reserpine to an albino rat its spontaneous motor activity begins to decrease. After 10—20 minutes it is sitting quite immobile. Auditory, tactile or olfactory stimuli activate the animal to movements of the normal pattern. The longer the time after injection the stronger are the stimuli required to provoke activity. After 20—30 minutes very intense activation is required to bring about adjustments of posture after which the animal immediately returns to its akinetic state. In this state the rat assumes a posture of general flexion. If gently placed on its back it will be immobile with the limbs flexed. The forelimbs are adducted at the shoulder joints, the elbows, the wrists and the metacarpophalangeal joints are flexed, but the interphalangeal joints stretched. The hind limbs are flexed at the hip and the knee joints but the feet are extended. The hind legs are adducted so that the feet are often crossed.

The grasp reflexes are intensely exaggerated both in fore- and hind limbs. A rat can be made to hang for half a minute from a rod grasped in the forefeet. The animal does not resist the imposition of bizarre postures and positions, where it remains until it shows obvious signs of fatigue and when thus forced to assume a new position it immediately returns into a fixed frozen attitude. The tail also shows a tendency similar to the grasp reflexes of the feet. It curves around objects touching its ventral surface and is used to help in the preservation of posture. If the animal is turned around its longitudinal axis the tail rotates as if active in a compensating reflex to regain equilibrium.

The muscular response to stretch is either constant or increases during extension but does not show a clasp-knife reaction. A tremor is often seen *in the fore- and hindfeet of a rat lying on its back. It is rapid and* is palpable in both limb and trunk muscles. When a muscle is stretched the rapid tremor rhythm can be recognized as an oscillation in the muscular resistance to elongation that resembles the cogwheel phenomenon of human parkinsonism although it has a higher frequency.

Sometimes instantaneous convulsions of myoclonic type are seen

Comment

Parkinsonism is usually described by the classical symptom triad of akinesia rigidity and tremor A definition of parkinsonism in these terms however disregards the postural element DENNY BROWN (1961 1962) directs attention to the posture of general flexion that gradually develops during the course of the disease and which he calls flexion dystonia In its mildest form it is seen in the hands as an overflexion of the metacarpo phalangeal joints with extension of the fingers A gradual assumption of flexed posture of the upper limbs and later of the lower limbs is asso ciated with increasing flexion of wrists and metacarpophalangeal joints and extension of the feet Particularly as an end stage in paralysis agi tans and related states a persistent attitude of flexion of all limbs may occur the wrists overflexed and pronated the fingers extended the lower limbs flexed on the abdomen and the feet stiffly plantar flexed In post encephalitic parkinsonism the postural changes may be asymmetrical or restricted to separate parts of the body The increasing muscular ac tivity at rest following the progression of the disease appears electro myographically as a tonic discharge of increasing numbers of motor units This tonic muscular hyperactivity with a flexor predominance which underlies parkinsonian posture is determined not only by the local joint position deciding the length of the studied muscle but also by the gen eral position of the limbs trunk and head A complete muscular inactivity can often be reached when the patient is supported in an optimal position and is instructed to relax (BUCHTAL and CLEMMESEN 1946) Some of the motor units still active in a state of complete relaxation are shown to be influenced by labyrinth and neck reflexes and can be silenced by manip ulating the head and neck (DENNY BROWN 1961 1962)

The changes of posture induced by neuroleptics in humans and animals show striking similarities to the flexion dystonia described by DENNY BROWN The increasing degrees of extrapyramidal disturbance in intensi fied neuroleptic treatment appears to correspond to the stages of devel opment of dystonia

In the last chapter of this study the neuropharmacological similarities between the reserpine syndrome and Parkinson s syndrome will be fur ther discussed

Chapter VI

Activity of muscle efferents in reserpinized rats

Introduction

The action of neuroleptic drugs on stretch reflex mechanisms has been studied in different preparations. In spinal animals reserpine was reported to suppress the tendon jerk and chlorpromazine to reduce tendon jerk and monosynaptic reflex amplitudes (PRESTON 1956, SILVESTRINI and MAFFII 1959, DE SALVA and OESTER 1960).

In the intercollicularly decerebrated dog chlorpromazine reduces extensor hypertonicity and hyperreflexia (SHEATZ 1955). HENATSCH and INGVAR (1956) found that chlorpromazine abolishes the "γ rigidity" of intercollicularly decerebrated cats but not the "α rigidity" after anaemic decerebration. The activity of γ motor axons is suppressed by chlorpromazine in the intercollicular preparation. SCHNEIDER *et al.* (1955) claimed that the monosynaptic reflex amplitudes of intercollicular decerebrate cats are increased after reserpine injection.

In intact cats anaesthetized with ether or nembutal chlorpromazine depressed and reserpine increased monosynaptic reflex amplitudes (EDISEN 1958). The tendon jerks of intact cats anaesthetized with chloralose were reported to be suppressed by both chlorpromazine and reserpine (SILVESTRINI and MAFFII 1959). Chlorpromazine injected to intact curarized cats was reported to augment the monosynaptic reflex amplitudes (STERN and WARD 1962).

All these methods involve decerebration or use of drugs that abolish the rigidity induced by neuroleptics.

An analysis of the stretch reflex mechanisms of experimental parkinsonism requires a technique that permits recording of the spontaneous and reflex discharge of single motor axons in the presence of the motor phenomena characterizing the neuroleptic and parkinsonian syndromes. In search for a preparation making possible electrophysiological analysis of neuroleptic drug action without the use of drugs obscuring the motor phenomena of the neuroleptic syndrome, the tail muscle innervation in the rat was studied. The segmental tail muscles of the rat were described

morphologically in chapter II Their innervation was analysed with electro physiological methods in chapter IV The third chapter presented the techniques developed for the basic investigation of muscle innervation as well as for this study of experimental neurology

Results

Reserpine effect on γ fibre activity

An incision in the tail was made to expose the muscle nerves and the rats were left to recover after the rapidly eliminated fluothane anaesthesia Two pairs of recording electrodes 2 5—5 mm apart were attached to a segmental tail muscle nerve which was crushed close to the muscle in order to eliminate afferent activity

The efferent potentials passing the two electrode pairs could easily be identified by the conduction time as γ fibre potentials of 10—20 m/sec conduction velocity and α fibre potentials of 20—50 m/sec velocity (Fig 9) The activity of the single γ efferent in the nerve could be accelerated by visual auditory tactile or rotatory stimuli The α efferents had a higher threshold for activation by external stimuli than the γ efferents Because of the constancy of the rotatory response rotation was used as the routine activation procedure The reflex activation of muscle efferents by rotation of the trunk of the animal may originate from receptors at the base of the tail It was difficult however to elicit similar reflex activity by manipulation of the tissues at the base of the tail Limb movements were observed simultaneously with the reflex changes of muscle efferent activity in the tail and may indicate a vestibular origin of the rotatory effects on motor axon activity

The calf muscle electromyogram of an awake rat before reserpine treatment was silent at rest and only showed weak reflex responses to repeated dorsiflexions of the foot Reserpine was injected intravenously in the rats After 7 mg/kg reserpine injected intravenously the reserpine syndrome appeared 10—20 minutes after the injection after 3 mg/kg the latency was 30—40 minutes Rigidity was recognized in the calf muscles on dorsiflexion of the foot The muscle stretch caused an intense reflex activation of the calf muscle electromyogram This electromyographic response always followed dorsiflexion of the foot and often the motor units of the muscle responded synchronously at 20—40/sec a frequency corresponding to the spontaneous tremor

Simultaneously with the development of rigidity in the calf muscles the γ efferent became silent and could not be activated by rotation or

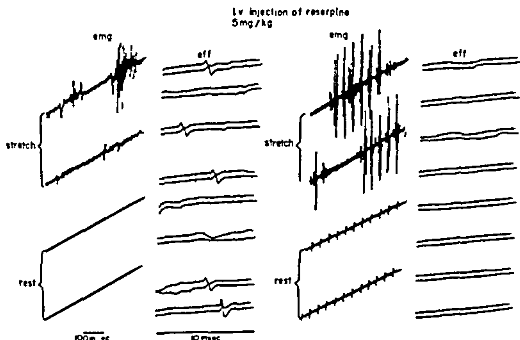


Fig 9 In an intact rat the gastrocnemius electromyogram (*emg*) was recorded at rest (*rest*) and when the foot was dorsiflexed (*stretch*) two consecutive sweeps for each are shown The activity was recorded in the efferent fibres of a segmental tail muscle nerve crushed distally to two pairs of recording electrodes placed 2.5 mm from each other the proximal pair connected to the upper and the distal to the lower oscilloscope beam (*eff*) The efferent discharge is recorded at rotatory activation Before reserpine injection the emg was silent at rest and gave a weak response to muscle stretch The last high amplitude asynchronous burst of activity was recorded during a movement of the limb The time lag of the single γ fibre potentials on the two beams corresponds to a 10 m/sec conduction velocity compared to 30—40 m/sec of the α fibre potential also demonstrated in the lowest trace Reserpine induces an increased electromyographic activity even at *rest* and on *stretch* a synchronous discharge appears The fusimotor discharge is completely abolished and cannot be activated by rotation

other stimuli (Fig 9) Occasionally spontaneous α fibre discharge appeared in the tail muscle nerve Usually it appeared first on rotatory or tactile stimulation and was not accompanied by activity in the γ efferents

Reserpine rigidity was thus accompanied by γ fibre inactivity Some observations indicated a lowered activation threshold of the α fibres in the reserpinized rat (cf Fig 10 and 12)

Reserpine effect on efferent and afferent activity of innervated muscle

By leaving the freed muscle nerve intact the activity of both efferent and afferent fibres could be recorded simultaneously. The activity of efferent and afferent axons was distinguished by the earlier appearance of efferent action potentials at the proximal and of afferent potentials at the distal pair of recording electrodes. The lateral segmental tail muscle studied was intact, left in its natural position and not stretched.

An experiment with this technique which makes it possible to relate the motor phenomena of the reserpine syndrome to changes of activity of all efferent and afferent nerve fibres of the innervated muscle is demonstrated in Fig. 10. Control recording after recovery from anaesthesia and before reserpine injection showed a spontaneous discharge of the γ efferent and the muscle spindle afferents. Rotatory activation provoked corresponding increases of the γ efferent and muscle spindle afferent discharge frequencies. The α efferents were inactive both at rest and on rotatory stimulation.

With a latency of 10—15 minutes after reserpine injection (6.5 mg/kg i.v.) the γ efferent and muscle spindle afferent activity began to decrease. At the same time a low frequency α efferent discharge appeared. Rotation increased the discharge frequency of all active fibres. Grasp reflexes were observed at this stage.

At the time when the γ efferent had completely ceased discharging, intense rigidity and cog wheel phenomena were perceived on dorsiflexion of the feet and the calf muscle electromyogram indicated a tonic hyperactivity both at rest and on passive extension and a spontaneous tremor. The α efferent in the lateral segmental tail muscle nerve was active only during an initial period and was then silent. On continued recording after the γ efferent discharge had ceased the muscle spindle afferent was active at a constant rate lower than that during γ efferent activity and rotatory stimuli did not affect the afferent discharge rate.

Reserpine effect on slow and fast motor units

Rats were enclosed in the plastic cylinder after they had been operated under fluothane nitrous oxide anaesthesia with an incision on the tail exposing the lateral and ventral segmental tail muscles of the left side. An hour after the operation 5—6 mg/kg reserpine was injected intravenously. When rigidity had developed a muscle was selected in which tonic electromyographic activity could be recorded by a pair of surface

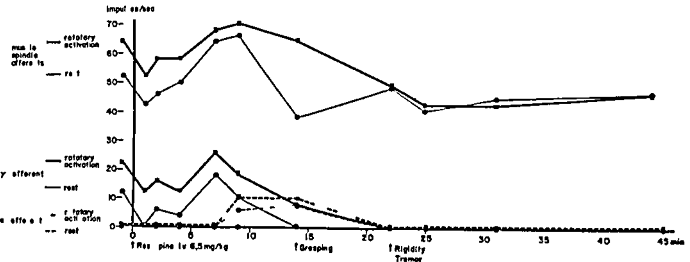


Fig 10 Plot of the activity of efferent and afferent fibres in the intact nerve to a lateral tail muscle following injection of reserpine The effect of reserpine on γ and α efferent and muscle spindle afferent discharge frequencies is shown at rest and on rotatory activation The appearance of grasp reflexes rigidity and tremor in the hindlimb of the rat is indicated The recording did not permit distinction between fast and slow α fibres

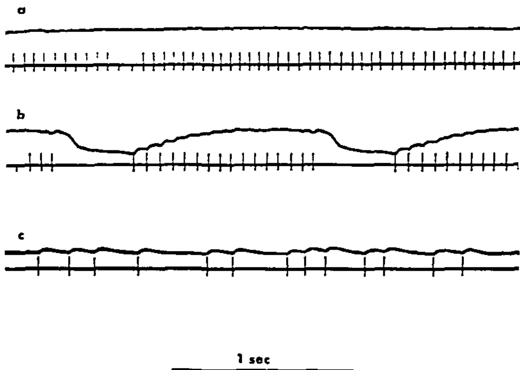


Fig 11 The activity of a single motor unit in a ventral tail muscle of a reserpinized rat is recorded mechanically by a strain gauge myograph attached to the tendon *(upper beam)* and electromyographically *(lower beam)* The contraction time of 45 msec indicates that it is a slow motor unit The motor unit is spontaneously active *(a)* Its activity can be stopped and started again by slight rotatory movements of the animal *(b)* In an appropriate position the unit discharges at low frequency demonstrating the single contractions *(c)*

electrodes Such activity was most often found in the ventral muscles of the more proximal exposed segments The strain gauge hook was connected to the short distal tendon disconnected from its insertion The mechanogram was recorded simultaneously with the electromyogram

The reflex modulation of motor unit activity during dorsiflexion of the hind foot and rotation of the trunk was studied The activity recorded in the muscle could be modulated so that few or single motor units were made to stop and start discharging with minimal rotations of the plastic cylinder containing the trunk of the animal Fig 11 demonstrates the tonic activity of a motor unit modulated in this way It has a contraction time of 45 msec and is thus a slow motor unit All motor units studied with this technique were slow motor units Due to their large amplitude the fast motor units would be easily recognizable even among several active slow units Not even with vigorous reflex stimulation was it pos

Fig. 12 The reflex and spontaneous activity of α and γ efferents was recorded before and after reserpine injection. The reflexes were recorded using two pairs of recording electrodes 3 mm apart on a coccygeal ventral root. The dorsal root was stimulated with a single test pulse (*T*) and with a conditioning pulse preceding the test pulse subthreshold for reflex activation (*C* + *T*). Shock artifacts indicated by arrows. The spontaneous efferent discharge was also recorded from the central root (*eff*). Calf muscle electromyogram (*emg*) recorded at rest (*two lower traces*) and at dorsiflexion of the foot (*two upper traces*) is silent before reserpine injection. Reserpine induces rigidity indicated by a spontaneous electromyographic activity at rest and a partly synchronized activity at muscle stretch. The γ efferent activity normally present is almost completely eliminated. A spontaneous α efferent discharge appears (*eff*). The α component of the spinal reflex (cf. Fig. 13) is enhanced after reserpine injection.

sible to activate fast motor units in the segmental tail muscles in reserpinized rats.

Reserpine effect on spinal reflexes

The assumption that reserpine produced an increased excitability of the α motoneurones was tested in a spinal reflex study. Rats were laminectomized and kept under light anaesthesia with nitrous oxide and the

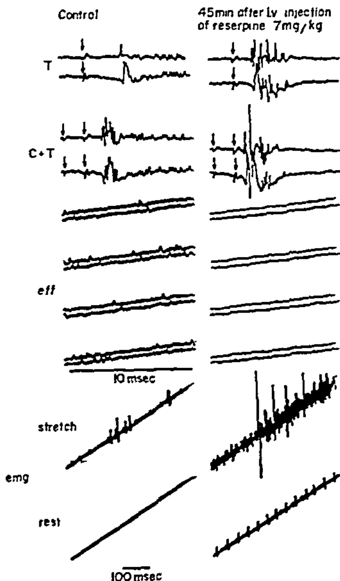


Fig 13 shows an experiment essentially similar to that of Fig 12 The electrode pairs attached to the ventral root are placed 9 mm apart The action potentials which are recorded in the ventral root on dorsal root stimulation are distinguished into α and γ fibre potentials by the difference in conduction velocity and spike amplitude The fast α potentials show a short and the γ potentials a long time lag between two pairs of recording electrodes The reflex response shown in *T* and *C+T* consists of one early α-component including the monosynaptic reflex and a late γ component Reserpine increases the α component reduces the γ-component of the spinal reflex suppresses the spontaneous γ activity at rest (*eff*) and induces rigidity in the hindlimb muscles shown at *rest* and in muscle *stretch* (*emg*) One α fibre is blocked at the distal pair of recording electrodes giving the changed shape of its action potential

remaining effect of Vandril given during the operation. By variation of the nitrous oxide dosage the level of anaesthesia in the spinal reflex experiments was adjusted so that the rigidity and muscle efferent activity of the former experiments was reproduced (Fig. 12 and 13). The left root pair of a coccygeal segment was placed on electrodes. The dorsal root was attached to a bipolar stimulation electrode and the ventral root was placed over two bipolar recording electrodes with 3—10 mm distance between the electrode pairs.

The spontaneous activity as well as the reflexly activated discharge was recorded in efferent fibres of the fine ventral root. The action potentials could be identified by the conduction time between the two electrode pairs. There was a continuous activity in a number of γ efferents in the resting state. Tactile and auditory stimuli elicited an increased γ efferent discharge and also gave rise to short bursts of α efferent activity. The reflex was composed of two groups of potentials. The first was a mono and polysynaptic reflex consisting of α potentials with a high spike amplitude. The latter group was a polysynaptic reflex with a latency of circa 3—7 msec and was formed by slow low amplitude γ potentials (Fig. 13).

Simultaneously with the electromyographic demonstration of reserpine rigidity at dorsiflexion of the foot the α component of the spinal reflex was strongly facilitated. At the same time the late γ component of the reflex was inhibited and the spontaneous activity of the γ efferents ceased (Fig. 13). An α discharge could be elicited by tactile stimuli or by dorsiflexion of the tail with a lower threshold than before reserpine. It sometimes appeared spontaneously (Fig. 12). No differentiation of fast and slow α axons was made in this spinal reflex study.

Discussion

Methodological problems of model syndrome analysis

A discussion on the neurophysiological analysis in animal experiments of a model syndrome simulating parkinsonism should consider three methodological criteria. 1. The model syndrome should present a close analogy to its clinical correlate. 2. The neurophysiological technique should not interfere with the motor phenomena to be studied. 3. The electrophysiological technique should give access to the neuronal mechanisms of motor phenomena.

The actions of neuroleptic drugs in the *spinal* preparation are of little significance for the analysis of neuroleptic rigidity. Too many of the

supraspinal projections influencing the spinal reflex pattern in the intact central nervous system are disregarded

The intercollicularly and anaemically *decerebrated* animals present the two basic γ and α mechanisms of rigidity the first being dependent on an intact γ loop the latter set up by direct α motoneurone activation by passing the γ loop The chlorpromazine and reserpine action suppressing the intercollicular but not the anaemic decerebrate rigidity has been taken as a basis for interpretations of the therapeutic actions of these drugs and for hypotheses on the pathophysiology of clinical spasticity and rigidity (SHLATZ 1955 HENATSCH and INGVAR 1956 STERN and WARD 1962) Supracollicular structures are known however to play an essential role in parkinsonism and the differences in motor manifestations between the anaemically decerebrated preparation and the parkinsonian syndrome are obvious Since this preparation cannot be regarded as closely analogous to the parkinsonian syndrome it is not reliable as a model or tool in the investigation of parkinsonism

General anaesthetics abolish the motor phenomena induced by neuroleptic drugs at all levels of anaesthesia but the most superficial The effects of neuroleptic drugs in preparations where the rigidity and tremor are not present are without significance for the analysis of the model syndrome

Curarization may make possible the study of the functions of the intact central nervous system without general anaesthetics A method of investigating the stretch reflex mechanisms of rigidity that includes curarization however abolishes the rigidity and introduces a factor of uncertainty The finding of an increase in monosynaptic reflex amplitudes after chlorpromazine injection in the curarized cat and the reduction in γ efferent activity demonstrated by HENATSCH and INGVAR in the intercollicular decerebrate preparation were taken as a basis for the hypothesis of a relative depression of γ activity and heightened α activity in Parkinson's disease (STERN and WARD 1962 1963)

The very long and fine nerves supplying the segmental rat tail muscles give a unique and ideal electrophysiological access to the individual nerve fibres innervating the muscles (cf chapter IV) *without* any *pharmacological interference with* the motor functions of *the intact nervous system* The motor phenomena of the reserpine syndrome were recorded electromyographically simultaneously with the spontaneous and reflex activity of individual motoneurone axons and motor units of different types The activity of efferent and afferent axons could be recorded simultaneously in the intact muscle nerve

Efferent muscle innervation in the reserpine syndrome

It has been a constant finding that reserpine decreases the γ efferent activity. This has been found not only on recording from the nerves to tail muscles but also on recording from ventral roots. The rigidity developed in parallel with the decrease of γ activity but did increase also after cessation of γ activity. Normally rotatory stimuli are very effective in activating the γ efferents but after reserpine the rotatory stimuli became ineffective. Simultaneous recording from muscle spindle afferents (Ia) showed that reserpine decreased their discharge frequency and abolished the acceleration that normally is evoked by rotatory stimuli.

When the γ activity ceased after reserpine administration there sometimes occurred a resting discharge in α efferents. This was found only occasionally in the nerves to tail muscles but more often in the ventral roots. The threshold for activation of the α efferents whether from rotatory stimuli, tactile stimuli or from dorsiflexion of the tail was very much lowered.

Recording of the contractions in the ventral segmental tail muscles revealed slow contractions in the motor units activated after reserpine injection. This was found both for the spontaneously discharging and the reflexly activated motor units. Hence this investigation has demonstrated hyperactivity in slow α motoneurones. The spontaneous tonic slow motor unit activity is found predominantly in the flexor muscles of the tail. It is particularly interesting that the discharge can be accelerated or inhibited by rotatory stimuli, which effect may be ascribed to vestibular reflexes.

In summary, in the normal rat the γ activity is the dominating efferent event; after reserpine the γ activity is reduced and the activity of slow α efferents is increased.

The hypothesis of a selective facilitation of the α motoneurones was verified in a spinal reflex study in which the same changes of muscle efferent activity and motor phenomena induced by reserpine were found as in the former experiments. An early α component including the monosynaptic reflex and a later γ component were identified in the reflex response recorded in a coccygeal ventral root on dorsal root stimulation (cf. HUNT and PAINTAL 1958). After reserpine injection the γ component was completely eliminated and the α component of the reflex was strongly increased. It is likely that the results reflect the changes in non-anaesthetized rats. The spinal reflex change may be described as a shift of facilitation from γ- to α-motoneurones. The rigidity recognized by palpation as muscular resistance to stretch and demonstrated electromyogra-

phically by increased activity is classified as an α rigidity It seems likely that the reflex hyperexcitability refers to the slow α motoneurones The fast α motoneurones may however show increased excitability A fast tremor palpated in several muscles was clearly perceived when the foot was dorsiflexed by the finger It could be recorded as a synchronized discharge in the gastrocnemius soleus electromyogram The frequency of this synchronous motor unit activity was 20—40/sec (Fig 9 and 12) At these frequencies the slow motor units produce a smooth fused contraction It is consequently assumed that the tremor is produced by fast motor units The finding suggests that there is an increase in the excitability also of the fast α motoneurones This question requires a further analysis of the stretch reflex activation of fast motor units The possibility suggested by BESSOU *et al* (1964) that the dynamic sensitivity of muscle spindles is influenced by branches from slow α motor axons might be of relevance for the stretch reflex activation of fast motor units

Summary

The γ efferents to the muscle spindles are inactive and the slow motor units are tonically hyperactive in the presence of akinesia rigidity and tremor in rats treated with reserpine It is concluded that the reserpine rigidity is an α rigidity Indirect evidence ascribes the reserpine tremor in the rat to phasic hyperactivity appearing in repetitive synchronous bursts of motor units probably having fast contractions

Chapter VII

General discussion

The segmental tail muscles of the rat are small enough to permit mechano-myographic recording of the individual contractions of all its motor units. They demonstrate a muscle organization with one fast and one slow group of motor units. The slow motor units have a high resistance to fatigue and are likely to be active in tonic muscle contractions.

The extrapyramidal syndrome induced by reserpine reproduces the motor symptoms of postencephalitic parkinsonism. This syndrome in animals is presented as a model or tool in the investigation of parkinsonian neurophysiology. Its appearance after reserpine administration is accompanied by fusimotor inactivity and a tonic discharge of slow motor units. The excitability of γ motoneurones is decreased and that of the α motoneurones is increased after reserpine administration.

Monoaminergic transmission, reserpine and parkinsonism

The application of these neurophysiological findings to clinical parkinsonism was tested neuropharmacologically and discussed by Roos and Steg (1964).

Increasing evidence indicates that the monoamine metabolism is disturbed in parkinsonism. In the normal brain, nucleus caudatus and putamen have high dopamine concentrations whereas only small amounts are found in other brain structures. The content of dopamine in corpus striatum of parkinsonian patients is markedly reduced. The synthesis of dopamine is supposed to be impaired in parkinsonism. Also the concentrations of other brain monoamines are reduced. L-3,4-dihydroxyphenylalanine (L-DOPA), the precursor of dopamine and noradrenaline, reduces the akinesia and rigidity of parkinsonian patients. 5-hydroxytryptophan (5-HTP), the precursor of 5-hydroxytryptamine, is reported to reduce mainly the parkinsonian tremor.

Reserpine blocks the uptake of monoamines in the stores of the nerve terminals supplying the monoaminergic synapses with transmittors and

lowers the content of physiologically active monoamines in the brain. An inhibited transmission in the monoaminergic synapses in some regions of the brain thus seems to be the common factor in parkinsonism and the reserpine syndrome. The akinesia, rigidity and tremor induced in humans by reserpine is reduced by L-DOPA. L-DOPA also reverses the reserpine effects in animals.

Using the technique presented in this study ROOS and STEG demonstrated that the reversal of the reserpine syndrome by L-DOPA is always accompanied by a simultaneous reappearance of the spontaneous γ efferent discharge and a reduction of the α motoneurone excitability to the pre-reserpine level. The same was found after injection of 5-HTP.

The result confirms the finding that the reserpine syndrome is linked to a reduced γ and an increased α motoneurone excitability. The finding further establishes a connection between the neurophysiological mechanism of the extrapyramidal motor phenomena and monoamine action. This experimental demonstration of L-DOPA action on the reserpine syndrome closely parallels the L-DOPA effects in humans pre-treated with reserpine and in parkinsonian patients.

The analogy in motor symptoms and in monoamine neuropharmacology makes it unlikely that parkinsonian pathophysiology differs considerably from the mechanisms revealed in the electrophysiological analysis of the reserpine syndrome.

Efferent muscle innervation in parkinsonism

There are a great number of clinical studies relating parkinsonian motor symptoms to functions of supraspinal structures. Few, however, consider the role of spinal reflex mechanisms in parkinsonian symptomatology. A pathophysiological understanding of the parkinsonian syndrome presumes knowledge of the segmental mechanisms modulated by influences from supraspinal projections to muscles. The stretch reflex functions basic in motor physiology are apparently changed in parkinsonism. The methodological difficulties in studying the stretch reflex mechanisms in humans are the background to the present confusion about reflex pathophysiology of parkinsonism. The information on parkinsonian stretch reflex functions rests on data gained by three main types of clinical techniques: 1. Neurological examination of the changes after therapeutic de-afferentation of rigid limbs by surgical dorsal root transection. 2. The effects of selective nerve blocks on rigidity. 3. Electromyographic study of the myotatic and monosynaptic reflexes in hemi-parkinsonism

Dorsal root transection

Surgical transection of the dorsal roots was introduced in the treatment of spasticity by FOERSTER (1908). He suggested the application of the method in parkinsonism (FOERSTER 1911) and claimed that dorsal root section abolishes the muscular hypertonia of "pallidal disease" but did not present any material from parkinsonian patients (FOERSTER 1921). POLLOCK and DAVIS (1930) transected the dorsal roots of the rigid arm in a case of postencephalitic parkinsonism and found that immediately after the operation the patient had much less rigidity than before operation. Some weeks later a "contracture" developed in the biceps and the flexor muscles of the wrists. The contracture relaxed during sleep and when the head, habitually kept fixed towards the intact side, was turned towards the deafferented side. Intramuscular procain injection did not affect the contracture. FOERSTER and GAGEL (1932) confirmed the finding by demonstrating the same contracture after a similar operation in a parkinsonian patient.

The identical observations in these two cases demonstrate an initial reduction of resistance to passive stretch and a flexor contracture gradually developing during the first weeks after the deafferentation, whereas in man widespread resection of posterior roots for pain in the absence of disease of the nervous system is not followed by increased tone but by hypotonicity (POLLOCK and DAVIS 1930). POLLOCK and DAVIS concluded that a fundamental element of parkinsonian rigidity is proprioceptive in origin. They stated that the flexor contracture, obviously independent of proprioceptive inflow, was influenced by tonic neck reflexes.

Selective nerve blocks

WALSHE (1924) demonstrated that intramuscular procaine injection abolished or greatly diminished the rigidity of paralysis agitans without interfering with voluntary movements. WALSHE's interpretation was that the procaine had blocked the afferent nerve fibres in the muscle but left the motor nerve fibres unimpaired.

MATTHEWS and RUSHWORTH (1957 a, b, 1958; MATTHEWS 1958) found that procaine nerve blocks paralysed tonic stretch reflexes and tendon jerks in decerebrate animals but not the muscle contraction to stimuli applied proximal to the block. The small γ motor fibres were blocked "at a time when the large fibre potentials were only beginning to decrease". Procaine injected either close to the sciatic nerve or intramuscularly in parkinsonian patients resulted in "abolition of hyperactive stretch

reflexes while voluntary activity was preserved (RUSHWORTH 1960, 1961). RUSHWORTH assumed that the action of procaine had been selective to the γ motor fibres and concluded that parkinsonian rigidity is due to an overactivity of the γ system that produces oversensitization of muscle spindles to stretch.

The finding of an elimination of rigidity but not of voluntary innervation for a period after the injection of procaine intramuscularly or close to a nerve may have justified the interpretation that a γ rigidity was abolished by a selective blocking effect on the γ fibre. Two alternative interpretations are possible however. The first is based on the assumption of a clearcut selectivity of procaine for finer fibres, which means that at a given moment all fibres finer than a certain calibre are blocked but no larger fibres. If parkinsonian rigidity is due to slow motor unit hyperactivity and the slow α fibres are blocked but not the fast, the rigidity may be abolished with maintained voluntary muscle control and without significant decrease of muscle tension on electrical stimulation proximal to the block. Another more likely alternative is that no such selectivity is reached when the procaine spreads in the muscle or penetrates into the nerve trunk. This interpretation gets support in GASSER and ERLANGER's (1929) study where they found that procaine in general blocks small fibres before large ones but stated that "the block is not effected with any precision". If the block affects some large fibres while other are intact, a decrease of proprioceptive input through Ia fibres may diminish rigidity, while the voluntary innervation through intact α fibres is still preserved.

This method of indicating human muscle spindle function thus cannot be taken as a basis for an assumption of different stretch reflex mechanisms in parkinsonian and reserpine rigidity.

Myotatic and monosynaptic reflex amplitudes

Myotatic reflexes elicited by tendon taps and recorded electromyographically are increased in amplitude during JENDRASSIK reinforcement in the healthy side but not in the rigid side of a hemiparkinsonian patient (HASSLER 1956). The increase in reflex response in the normal side was ascribed to increased muscle spindle sensitivity secondary to γ fibre activation (SOMMER 1940, PAILLARD 1955) and the absence of this effect in the rigid side was interpreted to indicate a γ system inactivity in parkinsonism. In a similar study STRUPPLER and FLEISCHHAUER (1955) found lower myotatic reflex amplitudes in the rigid compared with the healthy side in hemiparkinsonism, whereas the electrically elicited monosynaptic

H reflexes (HOFFMAN 1922, MAGLADERY *et al* 1950) were bilaterally equal. The finding was ascribed to a low muscle spindle sensitivity in rigidity due to reduced fusimotor activity or increased extrafusal contraction.

This interpretation corresponds to the afferent inactivity demonstrated in the reserpinized rigid rat. However, the differentiation of fast and slow motor units and the distinction between dynamic and static spindle sensitivity makes it unlikely that the reflex response to quick tendon taps will give reliable information on the participation of muscle spindles in tonic stretch reflexes.

Conclusions

The neurophysiological analysis of the reserpine model syndrome provides a theory of parkinsonian pathophysiology which can be applied in the interpretation of the neurological observations.

The observation of a muscle contraction in deafferented limbs of parkinsonian patients is well explained by the theory of a primary increase in α motoneurone excitability during γ system inactivity.

It is most likely that the distinction into slow and fast motor units is a general feature of mammalian muscle organization and that the slow motor units are considerably more resistant to fatigue than the fast ones (cf chapter IV). If so, the theory based on the reserpine model syndrome analysis ascribes the steady muscle contraction underlying the fixed flexion posture of parkinsonism to a tonic hyperactivity of the slow motor units.

This inference is supported by the observation that changes in the position of head and body in space influences the tonic muscle contraction underlying the posture of parkinsonian patients and reserpinized animals. The influence of labyrinth and neck reflexes on the tonic discharge of motor units in parkinsonism demonstrated by DENNY BROWN corresponds to the reflex modulation of the tonic activity of slow motor units found on rotation of the reserpinized rat.

The theory of a selective increase in excitability of the α motoneurones in parkinsonism applies to the muscle resistance on passive stretch. Even in the absence of fusimotor activity the muscle spindles have a basic sensitivity. Passive muscle extension increases the discharge in the muscle spindle afferents projecting to the hyperexcitable α motoneurones and produces a pathologic tonic stretch reflex giving the resistance to stretch. Section of the dorsal roots abolishes this pathological stretch reflex.

The presented results do not permit a discussion on the changes of

reflex mechanisms giving the characteristic plastic quality of rigidity *often found in parkinsonian patients or on the pathophysiology of parkinsonian tremor* These problems will be taken up in separate experimental studies

Although the definite proof demands clinical methods for direct study of the different types of peripheral axons innervating muscle the electrophysiological analysis of the reserpine syndrome in animals in connection with neurological observation in parkinsonian patients after dorsal root transection provides sufficient evidence for the postulate that parkinsonian rigidity [illegible] hyperactivity of slow motor units during system inactivity

The criticism [illegible] of Professor Anders Lundberg has been invaluable for the [illegible] program

Professor [illegible] introduced me to the field of neurophysiology and encouraged me [illegible] new knowledge of muscle control to clinical disorders of tone [illegible] through Professor Tore Broman's early interest in the experimental [illegible] program I was given the opportunity to be in full time work [illegible] advice of Professor Arvid Carlsson and the cooperation with Dr [illegible] given a necessary neuropharmacological basis for the work

Mr [illegible] Christin Johnsson and Mr Tomas Palm have given important technical assistance Dr [illegible] Jukes Miss Helen Brogren Mrs Kerstin Stromwall and Miss Berit Nilsson were helpful in the preparation of the manuscript

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HUMAN SINGLE MUSCLE FIBER ACTION POTENTIALS

Extracellular recording during voluntary and chemical activation.
With some comments on end plate physiology and on
the fiber arrangement of the motor unit

BY

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UPPSALA 1964

GENERAL INTRODUCTION

Electromyography i e recording of the electrical activity accompanying muscular contraction is an extensively used tool in the study of muscular physiology and has gained great importance in the diagnosis of neuromuscular disorders References to previous work in the field of human electromyography can be found in the bibliographies of ROSENFALCK (1961, 1963) Comprehensive reviews concerning the present status of electromyography are among others those of BUCHTHAL (1962) and WOOLF (1962)

The electrical activity can be led off either by surface electrodes fastened to the skin overlying the muscle or by intramuscular needle electrodes Recording with surface electrodes can only give a very crude picture of the electrical activity due to the considerable distance between the active fibers and the electrode A needle electrode comes closer to the muscle fibers Only the action potentials from the fibers within a limited area around the electrode are recordable But at voluntary contraction the muscle fibers are not independently discharging a number of muscle fibers—up to several hundred—are innervated by the same nerve fiber and constitute the motor unit Therefore the individual muscle fiber action potentials are usually not discerned but are mixed with other fiber action potentials from the same motor unit The proportion in which each fiber takes part in the generation of this *motor unit potential* is dependent on where in the motor unit the recording is made

BUCHTHAL GULD & ROSENFALCK (1957 *a*) consider that the fibers of a motor unit in the human muscle are grouped in small bundles of at most 30 fibers and these they term subunits The individual fiber action potentials in a subunit are considered to be highly synchronized and to give rise to the spike of the motor unit potential that is the diphasic components of short duration and high amplitude that are usually seen in the motor unit potential

There is a vast literature concerning the motor unit potentials but very little has been written about single fiber potentials Lately some investigations of the membrane resting potentials in human muscle fibers *in situ*

measured by capillary electrodes, have been published (JOHNS 1960 NORRIS 1962 CREUTZFELDT 1962, RIECKER BOLTE & VON BUBNOFF 1963) and also recordings of the intracellular action potentials have been made (BERANEK 1961) However no study of human extracellular single muscle fiber action potentials *in situ* has been published On the contrary ADAMS DENNY-BROWN & PEARSON (1962 page 117) in their monograph Diseases of Muscle state Attempts to record single fiber potentials by the use of electrodes with a very small 'pore or active surface in man have met with little success for with active electrode tips below 50 μ in human muscle, movement artefact becomes magnified out of all proportion to the potential registered

In the present paper a method of recording extracellular single muscle fiber action potentials in voluntarily activated human muscle is described The method is based on the use of a needle multielectrode where the electrodes can ' look at the action potential generator from different sites Thus it is possible to select generators consisting of one single fiber

The method of action potential pick up and display are in many respects different from those used in ordinary electromyographic work and therefore they are described here in detail The major part of this work deals however with the criteria to be fulfilled by single muscle fiber action potentials The criteria have been deduced partly from the recordings of muscle fibers that were made to discharge spontaneously by chemical stimulation and partly from the study of composite motor unit potentials It is shown that action potentials fulfilling these criteria must be derived from single muscle fibers

From an analysis of the recordings has been concluded that the individual fiber potentials may also be discerned with ordinary concentric needle electrodes and that the spikes of the motor unit potential are usually generated by single muscle fibers This is contrary to the opinion of BUCHTHAL *et al* (1957 *a*) who consider the spike source to be the subunit and only exceptionally a single fiber The results obtained in this investigation *are not consistent with the concept of a subunit arrangement of the* fibers of a motor unit

The present method of single fiber recording offers a new possibility for the study of the physiology of the neuromuscular junction It is shown that the difference in the arrival time to the electrode of two fiber action potentials from the same motor unit is not constant from discharge to discharge There are always small variations about the mean time interval of an order of magnitude of 10–30 μsec so that consecutive composite action potentials are of slightly different shape the so-called *action potential jitter'* The jitter

phenomenon has been chiefly attributed to variations in the time delay in the neuromuscular junction It does not seem to have been described in the literature The absence of jitter has proved to be the most important criterion for single muscle fiber recordings

With the multielectrode method the muscle fiber orientation and the direction of the propagation can also easily be determined and this has been used here for delineation of the end plate zone

A short communication concerning single muscle fiber recording and propagation velocity determination has been published previously (EKSTEDT & STALBERG 1963)

SURVEY OF THE LITERATURE

Current concepts of the motor unit

The motor unit is considered as a physiological entity since the work of LIDDELL & SHERRINGTON (1925) who briefly defined it as the motoneurone-axone and its adjunct muscle fibers

According to most authors all the muscle fibers of a motor unit are simultaneously active but it has also been claimed that only a part of the fibers of a motor unit are active at the same time RALSTON & LIBET (1953) consider that the degree of stretch in the muscle determines the number of active muscle fibers in a motor unit This opinion has been neither supported nor refuted in subsequent literature There is also the possibility that at fatigue there may be a partial neuromuscular block so that only part of the fibers are then active (BROWN & BURNS 1949)

The number of muscle fibers per motor unit in different human muscles has been investigated by FEINSTEIN LINDEGARD NYMAN & WOHLFART (1955) and CHRISTENSEN (1959) who divided the total number of muscle fibers in a muscle with the total number of motor nerve fibers Some examples of the values thus obtained are first lumbrical 108 first dorsal interosseus 340 tibialis anterior 562 gastrocnemius 1934 (FEINSTEIN *et al*) rectus superior oculi 23 opponens pollicis 13 brachial biceps 163 sartorius 300 rectus femoris 305 semitendinosus 713 (CHRISTENSEN)

However the values found in these two investigations are based upon an assumption that 60 per cent of the large nerve fibers in all muscles are motor fibers which in turn is based upon the investigations of the nerves to the cat anterior tibial and gastrocnemius muscles made by REXED & THERMAN (1948) The assumption was also supported by the findings in the nerve to a human gastrocnemius muscle with practically complete de-efferentiation due to poliomyelitis (FEX & WOHLFART (unpublished) quoted by FEINSTEIN *et al* 1955) It is however unlikely that the proportion of motor nerve fibers will be equal for such functionally different muscles as the gastrocnemius and the precision muscles of the hand or the external eye muscles

The values given by both FEINSTEIN *et al* (1955) and CHRISTENSEN (1959) are based upon one single determination for each muscle except for a few where two muscles have been used Therefore nothing can be said about the variations between different individuals The calculations are also mean values for the respective muscle and nothing is known of the deviation about the mean in the individual muscle Such a dispersion has been shown by ECCLES & SHERRINGTON (1930) who found considerable differences in contraction force between different motor units They also found that in one and the same nerve the motor nerve fibers of large diameter gave rise to a more intense peripheral branching than did nerve fibers of lesser diameter and this they considered an indication of the existence of motor units of different sizes However this latter opinion has been strongly opposed by FERNAND & YOUNG (1951) who consider that there is no correlation between nerve fiber diameter and the size of the motor unit This conclusion has been drawn from comparisons between different muscles with a different mean size of the motor unit

It is a well established fact that there is a certain order of recruitment of the motor units At low contraction force there are only a few motor units active at a slow discharge rate On increasing contraction more and more motor units are recruited and the individual motor unit discharge rate is increased up to about 30–40 discharges/sec (BIGLAND & LIPPOLD 1954)

Normally the motor units discharge entirely asynchronously but especially during fatigue there may be a tendency for a grouping of the motor unit potentials and this has been interpreted as a synchronization process between the moto neurones (e g ADRIAN & BRONK 1929 BUCHTHAL & MADSEN 1950 BIGLAND & LIPPOLD 1954 LIPPOLD REDFEARN & VUČO 1957) However TAYLOR (1962) considers this grouping to be a purely chance appearance without biological significance and found no true tendency to synchronization of moto-neurone activity

The motor unit potentials recruited at increasing contraction were found by KUGELBERG & SKOGLUND (1946 *a* *b*) to be of higher voltage the greater the contraction force exerted The order of recruitment of motor units was found to be the same at voluntary contraction and at electrical stimulation of the nerve by slowly rising currents The motor units active at slight contraction were considered to be smaller i e to have less fibers than those recruited at higher contraction strength These results also implied that small motor units should be innervated by large nerve fibers (having a lower threshold for electrical stimulation than smaller nerve fibers) This is contradictory to the findings of ECCLES & SHERRINGTON (1930) see above

BUCHTHAL, ERMINIO & ROSENFALCK (1959) did not find any systematic difference in 'territory' and 'fiber density' (see below) in the motor units already active at slight contraction and in those that appeared at stronger contraction. This they interpreted as due to the motor units being of equal size.

The size distribution of the muscle fibers in different human muscles has been extensively studied by, among others, SCHWALBE & MAYEDA (1890). The diameters range from 10 to 100 μ. Smaller muscles used in fine precision movements, e.g. the external eye muscles or the small muscles of the hand, have a smaller mean diameter than, for example, large muscles of the leg used more for developing force. In the brachial biceps muscle—the *muscle most used in the present investigation*—BUCHTHAL, GULD & ROSENFALCK (1955 *a*) found a mean diameter of 48 μ with range 25–85 μ.

However, nothing is known about the size distribution within the individual motor unit, i.e. if there is the same size distribution within each *motor unit, as in the whole muscle, or if there are motor units composed of* predominantly small or large fibers.

It is probable that there is some sort of functional differentiation between different motor units, as shown in the cat (e.g. GRANIT, HENATSCH & STEG 1956) where there are 'tonic' and 'phasic' moto-neurones with clearly different properties. In animal muscle, differing function is also reflected in the muscle fiber structure (e.g. HESS 1961); however, such differences have not been found in human muscle fibers (COERS & WOOLF 1957). The question of the functional differentiation in human muscle seems, at present, to be open. A review of the problem has been presented by LUDIN (1961).

It seems to be well established that the individual muscle fibers extend throughout the length of most muscles and that they are thus arranged in parallel (FEINSTEIN *et al.* 1955, COERS & WOOLF 1957). The gracilis and sartorius muscles are exceptions, the fibers also being arranged in series.

The topographical arrangement of the fibers in a cross section of the muscle has, however, been a matter of dispute. By dissection of the rabbit lumbrical muscle, FEINDEL (1954) found the fibers of a motor unit to be *scattered and separated by up to 8 fibers from other motor units.*

By studying the atrophied muscle fibers after a limited experimental denervation, VAN HARREVELD (1946) concluded that the fibers of a motor unit in the rabbit's sartorius muscle were widely scattered. KRNJEVIC & MILEDI (1958) stimulated single motor nerve fibers in a rat diaphragm preparation and found the muscle fibers innervated by that nerve fiber to be irregularly scattered over half the total area of the diaphragm.

WOHLFART (1959) considered there was an intermingling and a wide scattering of fibers from different motor units These conclusions were based upon direct microscopic studies of the branching of nerves in rat muscle and on analyses of denervated muscles from mice with experimental poliomyelitis (Theiler virus) He writes Strikingly often only some of the fibers within the terminal groups of motor fibers were degenerating while the remaining ones were intact If a terminal motor bundle were part of only one motor unit one would expect the entire bundle to be either degenerating or normal This implies a reversal of his earlier position i e that the muscle fibers of a motor unit were arranged in homogeneous bundles based upon studies of the atrophy pattern in spinal paresis in man (WOHLFART 1949)

NORRIS & IRWIN (1961) using single nerve fiber stimulation and intracellular recording in the rat peroneus longus muscle also found the fibers of a motor unit to be widely scattered In the animal muscle the intermingling and wide scattering of the fibers belonging to a certain motor unit seems thus to be well established

In man where more indirect methods of analysis have to be made BUCHTHAL GULD & ROSENFALCK (1957 *b*) BUCHTHAL, ERMINIO & ROSENFALCK (1959) have found the motor unit to be restricted to an approximately circular area the territory with a diameter of the order of 5–10 mm These conclusions have been founded upon electromyographic mapping of the cross sectional area of the muscle by means of a multielectrode with the electrodes spread over a distance of about 20 mm along the shaft of the needle The amplitude of the potential obtained in this way from the center of the motor unit was considered to be a measure of the fiber density of the motor unit The product of the maximum amplitude and the area of the territory is therefore considered to be a measure of the number of fibers in the motor unit (BUCHTHAL *et al* 1959) The territory was found to be enlarged in anterior horn cell disease (ERMINIO BUCHTHAL & ROSENFALCK 1959) and restricted in certain forms of myopathy (BUCHTHAL, ROSENFALCK & ERMINIO 1960)

DO CARMO (1960) investigated the territory of the motor unit in newborn *infants using the multielectrode method of Buchthal et al and found* approximately the same diameter of the territory as in adults in spite of the considerably lower total cross sectional area of the muscles and in spite of the same number of motor units and muscle fibers At present no explanation is forthcoming that accounts for this interesting finding

BUCHTHAL *et al* (1957 *a*) claim that the motor unit is subdivided the muscle fibers being grouped in bundles of 10–30 adjacent fibers termed subunits , approximately evenly distributed within the territory of the motor

unit The subunit gives rise to the spike of the motor unit potential i e a smooth clean spike potential of high voltage and short duration These conclusions have been founded on the following data

1 The spike potential was recorded with a multielectrode having 14 small electrodes spread over a distance of 2 5 mm in an opening at the side of a needle Spikes of high amplitude (more than 5 mV) were considered to have been obtained with the electrode positioned just outside the spike source Assuming an exponential voltage decrement with increasing distance the distance between the needle and the center of the spike source was calculated to be 85–150 μ and this was considered to be the radius of the spike source With a mean fiber diameter of 56 μ the spike source (the "subunit") was then calculated to contain 10–30 adjacent fibers No fiber from other motor units was assumed to be within the subunit

The smooth clean shape of the subunit spike in spite of its being generated by 10–30 fibers of different diameters and with different sites of innervation was explained as an effect of a mechanism of mutual interaction between adjacent fibers which equalized the propagation of the individual fiber action potentials

2 Sometimes two spike sources were found within the area scanned by the multielectrode The mean distance between the center of each pair of spike sources was found to be 0 7 mm (0 3–1 3 mm 15 recordings) Assuming an even distribution of the spike sources at a mutual distance of 0 7 mm within a territory of the motor unit of 5 mm (BUCHTHAL *et al* 1957 *b*) and assuming the motor unit to contain 1000 muscle fibers each spike source was calculated to contain 19 muscle fibers, this is of the same order of magnitude as found above

3 In spinal paresis due to amyotrophic lateral sclerosis an analysis of muscle biopsies reveals groups of 10–50 fibers with about the same degree of atrophy (WOHLFART 1949) Buchthal *et al* have repeated this histological investigation in mild cases of amyotrophic lateral sclerosis and found 10 2 ± 6 7 fibers per atrophic field The atrophic fiber groups were assumed to be subunits"

In later reviews of the motor unit organisation BUCHTHAL (1961 1962) refers to the subunit as containing 1–30 fibers'

No evidence for a subunit arrangement has been found in the animal experiments mentioned above by VAN HARREVELD (1946) FEINDEL (1954) KRNJEVIC & MILEDI (1958) NORRIS & IRWIN (1961)

An important factor when considering the possibility of recording single muscle fiber action potentials is also the degree of simultaneity between the different fibers of a motor unit A certain measure of this is the duration

of the motor unit potential. In the biceps muscle PETERSEN & KUGELBERG (1949) and BUCHTHAL, PINELLI & ROSENFALCK (1954 *b*) found mean values of duration of the motor unit potentials of about 7 msec, with extreme limits of 3 and 22 msec. With a duration of a single muscle fiber action potential of less than 1 msec (page 72) it can be concluded that the action potential from a certain fiber is not coincident in time with more than a part of the motor unit fiber potentials.

The duration of the motor unit potentials is due to differences in the arrival times of the individual fiber action potentials to the site of recording. These differences have been attributed by BUCHTHAL *et al.* (1955 *b*) mainly to the different sites of initiation of the impulses due to the extent of the innervation zone, and this was found to be about 40 mm in the biceps muscle for the same motor unit. They do not consider different propagation velocities of the muscle fibers and different propagation times in the terminal nerve endings to be important factors for the duration of the motor unit potential.

The topography of the terminal innervation of muscle has been extensively studied by COERS & WOOLF (1957) and CHRISTENSEN (1959). The end plate was located in the center of the muscle fiber (except for the long muscles, sartorius and gracilis, which have a scattered innervation) and there was generally only one end plate per muscle fiber. There was a double innervation in 2.3 per cent of the muscle fibers, but it was never observed that one muscle fiber was innervated from two different axons (COERS & WOOLF, 1957). Also physiological investigations provide no basis for a polyneuronal innervation (BROWN & MATTHEWS, 1960).

In man there seems to be a rather uniform shape of the end plates, both when comparing them in one and the same muscle and in different muscles. There seems to be a correlation between the size of the end plate and diameter of the muscle fiber. The mean diameter of the end plates in a series of human limb muscles was found to be $32.2 \pm 10.5\ \mu$, with a range of 10–80 μ (COERS, 1955, COERS & WOOLF, 1957).

Different types of electrodes used in human electromyography

Since ADRIAN & BRONK (1929) introduced the concentric needle electrode this has been the most frequently used type of intramuscular electrode for routine electromyography. It consists of an ordinary injection needle with a centrally mounted platinum wire exposed at the bevel point. The diameter of the central wire is often about 100 μ and the exposed surface has an oval

shape The outer diameter may vary between 0 35 and 0 65 mm for the electrodes that are commercially available Recording is made between the center core and the outer needle The physical parameters of concentric needle electrodes have been investigated by Buchthal, Guld & Rosenfalck (1954 *a*)

Instead of one two center cores may be used and by bipolar recording between these the area of uptake may be considerably restricted and the electrode accordingly made more sensitive in picking up potentials only from fibers in the immediate vicinity of the tip of the electrode (e g Landau, 1951 *b*)

Another commonly used type of electrode is a sewing needle or an entomological needle of outer diameter 0 3–0 5 mm lacquered except for the extreme tip The bare tip has a diameter of 25–50 μ These electrodes are easy to construct and give reliable results but are prone to develop small cracks in the insulation, creating false leading-off surfaces that can give rise to a misinterpretation of the electromyogram This type of electrode has been used extensively e g by Jasper & Ballem (1949) and in the extraocular muscles by Bjork & Kugelberg (1953 *a b*)

When electromyography is used for the physiological study of movement or of discharge patterns of the motor unit a very practical type of electrode consists of two fine insulated wires e g of tungsten introduced into the muscle by an injection needle that is subsequently withdrawn As the wires can be made very fine they can be kept in place in the muscle for a long time without the subject feeling their presence However once inserted their position can only be altered by retracting through the insertion channel created by the needle, where the muscle fibers are damaged This electrode type is not suited for the study of the action potential shape It has been used by Basmajian (1962) in determining the particular muscles that are concerned in specific movements (kinesiology) and further by Bigland & Lippold (1954) and Lippold Redfearn & Vuco (1957) in studies of the motor unit discharge pattern A technical description of such an electrode is given by Basmajian & Stecko (1962)

The type of electrode has a great influence on the shape of the action potentials recorded Comparative investigations concerning this have been made by Petersen & Kugelberg (1949) Pinelli (1949) Landau (1951 *a*) Lundervold & Li (1953) and Buchthal *et al* (1954 *a*)

An entirely new design of composite electromyographic electrode was introduced by Buchthal *et al* (1957 *a*) the needle multielectrode At the side of an injection cannula up to 14 electrodes were mounted Different multielectrode types were constructed One type had fourteen 50–100 μ leads arranged in a row of total length 2 5 mm and was used for the study

of the volume conduction of the spike of the motor unit potentials Other types had greater leading-off areas and longer mutual distances for the study of the territory of the motor unit (BUCHTHAL *et al* 1957 *b*) The great advantage of this type of electrode is that the action potential from one and the same generator can be led off from accurately known positions in a section of the muscle This type of multielectrode was used as the basic consideration in the design of the multielectrode used in the present investigation

A needle electrode design with one or several electrodes mounted in an opening at the side of a needle has been used by FLECK (1962) He has also introduced a type of needle electrode with electrodes mounted in two openings at opposite sides of a needle This type has been used in this investigation as well (page 19)

Glass capillary electrodes introduced through an injection cannula or an incision through the skin have been used for measurements of the muscle membrane resting potential by JOHNS (1960) NORRIS (1962) CREUTZFELDT (1962) and RIECKER BOLTE & VON BUBNOFF (1963) Only BERANEK (1961) seems however to have published any recordings of the intracellular action potential at voluntary contraction using such techniques After having inserted an injection cannula one of the fibers pressed into the opening of the bevel point by the intramuscular pressure and thus made immobile was pierced by a glass capillary electrode that was slowly advanced within the needle by a micromanipulator

Still another type of intramuscular electrode is the electromyographic myotome (BONSETT ABREU & RALSTON 1961) with which it is possible to take a muscle biopsy from the specific region where the electrical recording has been made

CHAPTER 1

METHODS

Recording

Electrodes

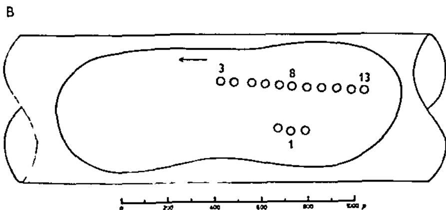
The 3+11 multielectrode

In an opening (0 5 × 1 5 mm) at the side of a stainless steel injection cannula 14 small round electrodes were arranged (Fig 1) The electrodes were the cut ends of 30 μ platinum wires each connected to one pin of a multipolar plug directly mounted on the needle The wires were embedded in heat hardened epoxy resin (Araldite® Ciba, Basle Switzerland) and ground flush with the side of the needle The electrodes were arranged in two rows one with 3 and the other with 11 electrodes The distance between the two rows was 200 μ and the adjacent electrodes were about 60 μ apart *The electrodes were thus spread over a distance of 600 μ along the* needle The face of the array and the adjoining part of the needle were ground flat. Thus, the needle had two diameters 0 5 and 0 55 mm

A small needle diameter was chosen so that the least damage was done to the muscle fibers upon insertion The condition of the point of the needle was found to be of fundamental importance It was confirmed many times that when the point was even slightly bent or was blunt it was very difficult to obtain action potentials of high amplitude probably due to damage to fibers close to the needle Therefore the point was reground under the microscope before each experiment first with number 3/0 emery polishing paper and then by strapping against smooth leather The bevel surface was *at the opposite side of the needle from the electrode array* so that those fibers pushed aside by the bevel point and perhaps compressed were not used for recording The subject was grounded by the shaft of the multielectrode No difference in the shape of the recorded action potentials could be detected when shifting from this way of grounding to a large skin electrode with the shaft disconnected from ground

The leads from the electrodes run close together for a length of 5 cm within the needle This fact was responsible for an inter-electrode capacitance of 8–10 $\mu\mu$F (page 29) for the different electrode combinations The





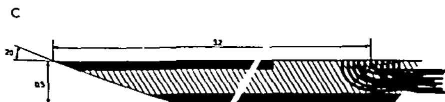


FIG 1 *The 3+11 Multielectrode A* Photograph of the multielectrode *B* The region with the electrode array magnified about 550 times redrawn from microphotographs *C* Schematic longitudinal section through the long electrode row

inter-electrode capacitance was further increased by 5–10 $\mu\mu$F in the input cable of the amplifiers (page 28) This gave rise to a capacitive cross talk of the signals between the different electrode leads The amount of charge transferred through a capacitive coupling is proportional to the rate of rise

of the voltage difference The cross talked signal between two electrode leads was usually of the order of 4 per cent (see Fig 36) The cross-talk was responsible for the inequalities in the action potentials from electrodes 1 and 8 seen in some of the recordings (e g Fig 10) Cross talk is discussed further on page 75

Not only the cross talk but also the noise level and the shunting of the higher frequencies by stray capacitances to ground are functions of the electrode impedance which must therefore be kept low It can be sub stantially reduced by a method of electrolytic treatment of the electrodes devised by BUCHTHAL *et al* (1957 *a*) Their method has been adapted in this work and was performed in the following way prior to each experiment The multielectrode was immersed in physiological saline The negative pole of a 4 5 volt battery was connected to one of the electrodes via a 0 1 megohm resistor The other pole was connected to a platinum needle also immersed in the saline bath The current 10–20 μA was passed through the electrodes for 10–20 seconds under microscopic control The procedure was repeated for each of the electrodes

The electrical properties of the multielectrode are further discussed on page 29

The positions and the distances between the centers of the electrodes were determined by microphotography A stage micrometer with 10 μ divisions (J D Moller Wedel in Holstein) was photographed at the same magnification and used for measurement at a total magnification of 2000 times The maximum error in the measurements was estimated to be less than ±2 μ

The electrodes were not positioned in the strict geometrical pattern that was originally intended The inter-electrode distances varied between 52 and 63 μ The greatest differences happened to be between the electrode combinations 7–8 and 8–9 which made these electrodes unsuitable for the special bipolar recordings (page 39) For these recordings electrode com binations 8–9 and 9–10 with the distances 61 and 63 μ respectively were used instead In all calculations and measurements actual distances were always used

The long electrode row happened to deviate 8° from the long axis of the needle and this had to be compensated for when making rotation adjust ments of the needle position

Damage to the electrodes or the surrounding insulator sometimes made re-polishment of the needle necessary If extensive such a re polishing could change the interelectrode position so that new microphotographic measure ments were necessary

The needle was sterilized in boiling water for 20 minutes

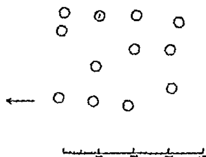


FIG 2 *The 3 × 4 Multielectrode* The electrode arrangement in the opening at the side of the needle redrawn from a microphotograph The arrow points towards the tip of the needle The general appearence of this multielectrode is the same as for the 3 + 11 multielectrode shown in Fig 1

The 3 × 4 multielectrode

Due to a constructional failure the electrode arrangement in this multi electrode became very irregular but just this property made it useful for some special applications There were three irregular rows with four round platinum electrodes of diameter 30 μ (Fig 2) in an opening at the side of a needle with diameter 0 55 mm The constructional and electrical properties were the same as for the 3 + 11 multielectrode

The Janus electrode

This electrode (Fig 3) was pointing in two opposite directions simultaneously and has accordingly been designated a "Janus electrode One platinum electrode of diameter 25 μ was mounted centrally in each of two openings at the opposite sides of a needle 0 60 mm in diameter This needle electrode was used for making simultaneous recordings from both sides of the needle the objective being to elucidate some problems concerning the motor unit organisation

A similar electrode has been described by FLECK (1962)

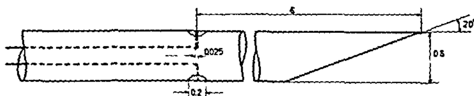


FIG 3 *The Janus electrode* Two round platinum electrodes mounted opposite to each other flush with the shaft of the needle

The concentric needle electrodes

The concentric needle electrodes used were types 13 K 51 and 13 K 03 (Disa Elektronik, Herslev Denmark) The center core was of diameter 100 μ the needle diameter 0 45 mm (13 K 51) or 0 65 mm (13 K 03) and the bevel angle 15°

The reference electrode

Recording was usually made between the electrodes in the multielectrode and a subcutaneous needle electrode as a reference The latter had a platinum surface of area 0 05 mm^2 exposed in the bevel surface of an injection needle

To make the reference electrode less liable to pick up activity from the muscle under investigation it was placed over the muscles antagonistic to it That the reference electrode was electrically indifferent was always checked at the time of the experiment

The cross talk could be considerably reduced by using one of the electrodes in the multielectrode as reference (electrode 3 or 13 in the 3+11 multielectrode) as used by BUCHTHAL *et al* (1957 *a*) Approximately similar cross-talked signals were then fed to both grids of the differential amplifier and cancelled or at least partially so However it was necessary to ascertain at each individual recording that the reference electrode did not pick up any activity (see BUCHTHAL *et al* 1957 *a*) and this proved to be tedious and time-consuming Therefore the subcutaneous reference has usually been preferred

Amplifiers

Each of the 14 electrodes in the multielectrode had its own cathode follower (capacity coupled) and the leads were surrounded by individual screens connected to the cathodes of the cathode followers (see ATTREE 1949 NASTUK & HODGKIN 1950) This arrangement with a multiplicity of cathode followers was chosen instead of using selectors for both lead and screen in the high impedance input circuits (BUCHTHAL *et al* 1957 *a*) as the wiring to the selectors inevitably introduced a considerable capacitance both to ground and between the leads This affected the high frequency response and increased the cross talk between the leads when using high impedance electrodes By means of six selectors each cathode follower output could be connected to any of the input grids of three differential amplifiers

The drawback with this arrangement was however the need for ac-

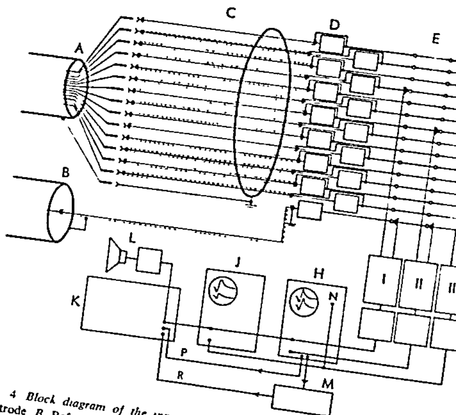


FIG 4 *Block diagram of the input circuitry and the experimental set up* *A* Multi electrode *B* Reference electrode *C* Input cable with separate screening for each lead *D* Input cathode followers with their outputs connected to the screens of the input leads *E* Selectors *F* Differential amplifiers *G* Attenuators *H* Oscilloscope used in the photographing recording *J* Monitoring oscilloscope *K* Ink jet recorder *L* Loud speaker with amplifier *M* Photography device governed by the oscilloscope *N* External trigger input from channel III *P* Signals marking the sweep of the oscilloscope *R* Signals marking the period during which the camera shutter is open

curately matched valves and other components By careful selection and adjustments it was possible to equalize the gain of the different cathode followers to within 0 15 per cent which corresponded to a common mode rejection of about 700 times (see page 29)

The 14 individually screened input leads had a common grounded screen The multicable of length 70 cm was flexible and light and had an outer diameter of 9 mm (Disa Elektronik Herslev Denmark)

The two amplifiers that were used for recording had identical properties to within 1 per cent They were capacity coupled without feedback The preamplifier parts were similar in construction to the Tetronix type 122

Low-Level Pre amplifier Attenuators made the amplification variable between 0 3 μV and 10 mV/cm on the oscilloscope in 22 steps with factor $\sqrt{2}$ The attenuation was frequency independent with an accuracy better than 1 per cent

The bandpass was variable stepwise between 0 2 and 40,000 cps (– 3 db) It was usually set at 60–40 000 cps (limited to 35 000 cps by the multi electrode see page 29)

The signals from two electrodes could be added in the amplifiers (see page 44)

The specifications for the amplifiers are given on page 28

The third differential amplifier had a limited frequency response but was only used for monitoring and triggering purposes

A block diagram of the input electronic circuitry and the experimental set up is given in Fig 4

Action potential display

In ordinary electromyography the potential sampling for the study of action potential shape is usually a purely random procedure In the diagnosis of for example myopathy where the pathological processes are known to be patchy within the muscle it may be necessary to search for a pathological area (KUGELBERG 1949) but once action potentials are picked up no adjustment of the needle for any optimum position within the motor unit is made The action potentials are usually displayed with free running sweep on the oscilloscope and photographed with the film slowly moving perpendicularly to the sweep direction (see e g BUCHTHAL 1957) The action potentials appear randomly at the different sweeps but can easily be identified for measurement

The present situation was however entirely different It was necessary to search for a certain position in relation to a muscle fiber and to keep the multielectrode in that position with high precision during the whole recording procedure It was also necessary to check the single muscle fiber criteria directly on the oscilloscope before recording This was not feasible with the action potentials randomly occurring at different sites on the oscilloscope screen usually together with several action potentials from other motor units The only possible way seemed to be to trigger the sweep by the initial positive phase of the action potentials as performed by BUCHTHAL *et al* (1957 *a*) Then all consecutive action potentials were displayed at the same position on the screen and were seen as a fixed pattern even at a discharge rate of only a few action potentials per second

When the initial positive phase of an action potential reached an optional

voltage the sweep triggered If several different action potentials were picked up by the electrode all of them that had a positive phase reaching the particular triggering level were displayed In order to select only one action potential the triggering level must be increased until only that action potential with the biggest initial phase remains on the screen If two action potentials had positive phases of the same voltage they could be separated by triggering the sweep not at a certain voltage but at a certain change of voltage i e the first time derivative of the voltage

Another possible way to separate two action potentials with about the same positive phase was to use the signals from another electrode for triggering where the action potentials from the two sources markedly differed in amplitude In general however the procedure of triggering favoured the action potentials of high amplitude if high and low amplitude potentials were picked up at the same time

A Tectronix (Beaverton Oregon U S A) dual beam oscilloscope type 502 with P 11 phosphor was used for recording A sweep of 20 or 100 μsec/cm was usually used To facilitate the identification of the two channels one of the beams could be intensity modulated by a 5–20 × 10³ cps A C signal

A second dual beam oscilloscope (Telequipment, type D 31 London Great Britain) was used as an aid for keeping the multielectrode in position while photographing the above mentioned oscilloscope

The screen was photographed by a 16 mm movie camera (Beaulieau R 16 Paris France) A special device made the camera advance one frame when a sweep had passed on the oscilloscope and kept the shutter open until the next sweep had passed and so on The camera thus followed the action potentials recording all up to a discharge rate of 4/sec At higher rates the camera photographed the first discharge to come after the refractory period of the camera device At a discharge rate of 20/sec every fifth action potential was thus photographed one on each frame For comparison of the shape of consecutive discharges of an action potential an optional number of discharges could be superimposed on one frame (see e g Fig 19) By altering the vertical position of the trace a few millimeters for each discharge a carpet of action potentials was obtained where the variations in time interval between two components could be conveniently studied (see Fig 23)

The number of the exposure and the time of exposure were indicated on electromagnetic counters which were also recorded on each frame By using a fast but nevertheless extremely fine grain negative film (Eastman Double X type 7222 Rochester N Y U S A) developed in Kodak D 76 developer for 12 minutes the beam intensity of the oscilloscope could be kept low In

spite of the small size of the image no loss of detail was noticed when it was projected for measurement at a magnification of 27 times corresponding to 1 5 times the actual size For this purpose a 16 mm film editor viewer was used (model Luxe Muray Paris France) which was modified by a larger hood and screen thereby producing greater magnification than the original and improving the linearity of the projected image The oscilloscope graticule was photographed on each frame and used as reference for the measurements The procedure of photographing and projecting therefore did not increase the uncertainty in the measurements (cf page 29) By cinematographic projection of the film in the viewer comparison of the shape of consecutive action potentials could be made in the same way as when directly inspecting the oscilloscope

For the study of the innervation frequency and rhythm the action potentials were also recorded on one channel of a three channel ink-jet recorder with a paper speed 1–10 cm/sec (Mingograph type 24 B Elema, Stockholm Sweden) Owing to the limited high frequency response of the recorder (600 cps) the action potentials were seriously distorted which was however not important for this application On the second channel each sweep of the Tectronix model 502 oscilloscope was signalled, and on the third channel there was a timing mark and a signal during the period when the camera shutter was open (Fig 5) Thus the same action potential could easily be identified on the oscilloscope photograph and on the ink recording

Loudspeaker display was invariably used as an indispensable aid in the positioning of the multielectrode and in monitoring the recordings

Calibration

For voltage calibration the built in 10 mV calibration pulse in the Tectronix type 502 oscilloscope was used This in turn was calibrated to within 1 per cent absolute accuracy with a model 44 Substandard Multi Range Meter (Electronic Instruments Ltd Great Britain)

A Philips (Einthoven Holland) Gated Timing unit Gm 8892/09 was used for *time calibration This delivered pulses at intervals of 100 μsec with an accuracy* of 0 1 per cent

The voltage and time calibrations were checked before each experiment but had to be reset only exceptionally

General experimental procedures

The detailed procedures will be dealt with in Chapter 2 in conjunction *with the discussion of the single muscle fiber action potentials* The following general remarks apply to all the special procedures to be described later

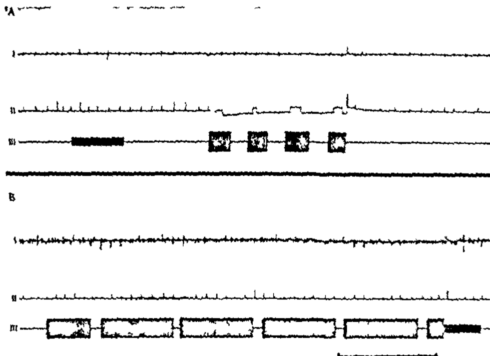


Fig 5 *Examples of ink recordings* Channel I records the action potentials Channel II records the instant when the sweep is triggered on the oscilloscope and channel III the period during which the camera shutter is open (broad markings) The narrow markings appear every 10th second In the recording *A* single sweeps have been photographed on the oscilloscope In the recording *B* two to eight consecutive action potentials have been superimposed on each oscilloscopic photograph

Note in *B* how the oscilloscope selects the action potentials from only one of the several sources that are active at the same time Calibration 1 sec

The electrode was usually inserted with the muscle under slight contraction and with the plane of the cutting edges of the tip parallel with the direction of the fibers This was considered to cause as little damage as possible When searching for action potentials an amplification corresponding to 50–100 μV/mm was used When a suitable action potential was found the amplification was adjusted so that the amplitude registered 3–6 cm on the oscilloscope A sweep of 100 μsec/cm was always used for searching Once an action potential was found the triggering level of the oscilloscope was adjusted to give a stable display on the oscilloscope with as little loss as possible of the positive phase of the action potentials The orientation of the action potential generator could be ascertained in the following way The position of the needle was adjusted for maximum amplitude on electrode 1 the center one in the row of three (Fig 1) Then the electrode in the long

row showing maximum amplitude was found by switching in one electrode after the other If the maximum was found elsewhere than at the center, then the fiber passed obliquely In this case the fiber orientation was noted and the needle re-inserted in the corrected direction Once the active fiber was positioned over electrodes 1 and 8 a further check was made by moving the needle slightly to and fro and watching the action potentials from elec trodes 1 and 8 When the needle was correctly positioned the two action potentials were equal in every phase of the movement and attained maximum amplitude simultaneously This was a very sensitive test making it possible to recognize a misalignment of less than 3 Deviations less than 10° could be corrected by gentle adjustments *in situ* When the long electrode row was perpendicular to the fiber the correct position as to the rotation of the needle was ascertained by making the action potentials from electrodes 1 and 8 equal They should then both be of maximum amplitude

The direction of propagation could be determined by noting which of the action potentials from electrodes 1 and 8 was leading and which was trailing Thus the innervation zone could be delineated (see page 32)

It must be emphasized that continuous readjustments of the needle posi tion usually had to be made It was therefore not possible to use any external fixation device for the multielectrode It proved to be best to hold the multielectrode by hand sometimes with the cable suspended so that no manual force was necessary to keep the needle in position

For monitoring the electrode position a bipolar recording was found to be a most sensitive aid Making the bipolarily recorded action potential of minimum size if possible zero meant that the muscle fiber passed strictly between the two electrodes in the bipolar pair The screen was photographed until it was ascertained that at least 3–5 of the photographs had been made with optimum position as judged from the bipolar recording

The action potential nomenclature used in this investigation is given in Fig 7

Temperature measurements

A constantan–manganin thermo-element was used One junction was en closed in an injection needle 0 5 mm in outer diameter near the tip and the other junction was placed in an oil bath thermostated at 35 00°C ±0 05 C The thermal e m f was measured by a D C microvoltmeter (Philips GM 6020 Eindhoven Holland) The accuracy of the method was estimated to be better than 0 1 C

Chemical stimulation

In order to be able to inject a solution for chemical activation of muscle fibers into a predetermined position in the muscle a special combined injection cannula and electrode was constructed In an opening at the side of a needle (diameter 0 6 mm) two 50 μ platinum electrodes were mounted at either side of the mouth of a glass capillary with inner diameter 150 μ (Fig 6) The solution was injected by a motor driven syringe connected to the needle via a long polyethylene tube delivering 7 6 μl sec of the stimulating solution The electrodes could be connected either to the output transformer of the stimulator or for recording to an amplifier By electrical stimulation this needle device could be positioned on the same fibers as the multielectrode some centimeters away After the injection of the stimulating solution the induced activity could be monitored by recording from the electrodes

A 0 1 *M* sodium citrate solution was used for stimulation advantage being taken of its calcium binding property Sodium citrate is readily available in sterile solution Chelating calcium by Versene (EDTA-disodium salt) *did not offer any advantage over the citrate solution*

Electrical stimulation

A double pulse stimulator delivering rectangular pulses of variable width frequency amplitude and mutual time difference was used The output circuit was a doubly shielded transformer with the secondary shield free from ground and connected to the shaft of the stimulation electrodes as described by BUCHTHAL *et al* (1955 *a*) in order to lessen stimulation artefact A pulse width of 130 μsec was usually used

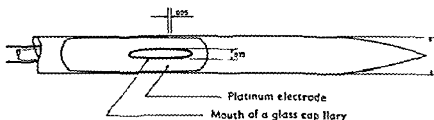


FIG 6 *The injection electrode* A schematic drawing showing the two platinum electrodes on either side of the mouth of the glass capillary mounted in an opening at the side of the needle The whole device is ground flush with the side of the needle

Electrical properties of the recording apparatus

Test equipment

A low frequency oscillator (Krohn Hite Model 420-A Cambridge Mass U S A) was used giving sine and square waves of frequency 0 4–50 000 cps The signals were attenuated to a voltage variable between 0 and 150 mV The output impedance of the attenuator was 100 ohms

The following procedure was used for feeding the signal through the multi electrodes The needle multielectrode was inserted through the walls of a rubber cup of volume 0 1 ml (the cap of an injection bottle) so that the electrodes were exposed at the inside The exposed part of the grounded needle stem was in sulated by wax that if necessary could also be applied to some of the electrode surfaces The cup was filled with normal saline The signals were fed to a pla tinum needle inserted into the cup in such a way that about 10 mm² of the needle was exposed at the inside which is about 10 000 times the electrode area Resistors and capacitors that were interconnected between the platinum needle and the signal generator were soldered directly to the platinum needle If neces sary to prevent mains interference the assembly was wrapped in aluminium foil

The measurements were made on the Tectronix type 502 oscilloscope

For resistance and capacitance measurements an ESI Mod 250 DA Impedance Bridge (Electro Scientific Industries Portland Oregon U S A) was used

Amplifiers and oscilloscope

The input impedance was measured by feeding a sine wave to the input cable of the cathode followers via a resistor The voltage drop measured on the oscilloscope at 50 cps with a resistor of 100 megohm was used for calculating the input resistance which was found to be 250 megohm By increasing the frequency until the voltage drop with a 2 megohm resistor was 3 db the input capacity of the input cable and the input circuits was found to be 10–15 $\mu\mu$F

The interlead capacitance was determined by feeding an A C signal to one lead of the input cable via a 0 1 megohm resistor and measuring on the oscil loscope the cross talked signal to the other leads that were at the multielectrode end of the input cable and grounded by 0 1 megohm resistances The interlead capacity was found to be 3–5 $\mu\mu$F

The frequency response was measured by feeding a 10 mV signal to the input cable of the cathode followers and measuring the peak amplitude on the oscil loscope The curve was flat between 1 and 20 000 cps and reduced 3 db at 0 5 and 40 000 cps

The rise time was measured by feeding a 10 mV square wave to the input cable of the cathode followers The waveform was square without overshoot The frequency was increased until the amplitude of the wave had decreased to 80 per cent A half period then corresponded to the 10–90 per cent rise time and this was found to be 8 μsec

The phase shift was found by feeding the input signal also to the x amplifier of the oscilloscope and watching the Lissajous figures Phase shift was a linear function of frequency with a delay time of 5 μsec for all frequencies within the flat part of the frequency response curve Thus there was no phase distortion (see TERMAN & PETIT 1952 p 275)

The nonlinear distortion was estimated to be negligible for signals below 150 mV for all frequencies as concluded from the study of the Lissajous figures

The common mode rejection was measured as the ratio between the voltage of the in phase signal and the out-of phase signal that gave the same output A 1000 cps square wave was used Common mode rejection for any combination of input cathode followers was about 700 times This also served as a check of equal amplification for the different cathode followers to within 0 15 p r cent

The noise level with short circuited input and full bandwidth was 2 μV RMS At timebase 20–100 μsec cm the tracewidth caused by the noise of the oscillo scope was approximately 6 μV

The non linearity of the sweep and the vertical amplifiers of the oscilloscope was less than could be measured with the present methods

The instability of the sweep the timebase jitter" i e the blurring of the oscilloscopic display due to different positions of consecutive sweeps when dis playing a recurrent waveform was not detectable

The accuracy of measurement on the enlarged photograph of the screen was estimated to be $\pm$1 mm For an action potential of amplitude 60 mm on the *enlarged photograph this added $\pm$2 per cent to the uncertainty in the amplitud* measurements

The multielectrodes

The interelectrode and electrode to ground insulation resistance was more than 500 megohm

The interelectrode capacity for all possible electrode combinations was 8 1–10 1 μ F

The electrode to ground capacity measured between each electrode and the needle was 14–16 $\mu\mu$F

The electrode impedance The electrode impedance is both frequency and voltage dependent (BUCHTHAL *et al* 1954 *a* BURKHARDT 1957) and proved to be very difficult to measure As the values obtained so far are uncertain they have been omitted

The noise level of the electrolytically treated electrodes was measured with the saline bath grounded and was found to be 10 μV RMS corresponding to a trace width at the oscilloscope of roughly 30 μV when using timebase 20–100 μsec/cm The noise had the gross appearance of and sounded like white noise i e had the frequency components approximately evenly distributed within the passband

Transmission characteristics of the multielectrode plus the amplifiers and the oscilloscope

The measurements were made as above The method for measuring the frequency response and the common mode rejection is essentially the same as described by BUCHTHAL *et al* (1954 *a*)

The frequency response curve was flat between 2 and 20 000 cps and reduced 3 db at 1 and 35 000 cps The values were the same for signals between 50 μV and 150 mV

The rise time 10–90 per cent was 12 μsec There was no overshoot in the recorded square wave

The phaseshift There was no appreciable phase distortion within the flat part of the frequency response curve

Common mode rejection was found always to be more than 200 times and usually in the order of 500 times for all frequencies within the passband

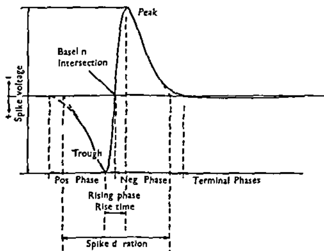


FIG 7 *Action potential nomenclature* "Spike denotes the fast positive–negative phases of a clean smooth action potential of the type shown It is only assigned to the action potential shape and is used irrespective of its being generated by one or several fibers This definition is in principal in accordance with that used by BUCHTHAL *et al* (1957 *a*)

As is conventional in neurophysiological work a downward deflection on the oscilloscope indicates a positive signal *Trough* and *peak* denote the points of maximum voltage for the positive and negative phases respectively Measurements of time interval between two spikes were always made between the baseline intersections

ACTION POTENTIALS DURING CHEMICAL ACTIVATION

THE SINGLE MUSCLE FIBER CRITERIA

Since the needle multielectrode was inserted blindly in the muscle the conclusions concerning the action potential generator must be deduced entirely from the recordings The 3 + 11 multielectrode was constructed to make possible recordings from the same generator at several sites of well known mutual relation Experimental data as to what is to be expected from the recordings of single muscle fibers *in man* are however lacking and therefore a method was sought to obtain a discrete fiber activity in the muscle Electrical stimulation by needle electrodes was tried but according to BUCHTHAL, GULD & ROSENFALCK (1955 *a*) the electrically elicited action potentials are generated by small groups of fibers and therefore this method was discontinued Diphasic fibrillation potentials found in denervated muscle were also investigated but as there is a controversy as to whether these potentials originate from single muscle fibers (DENNY BROWN & PENNYBACKER 1938) or from small groups of fibers (ROSENFALCK & BUCHTHAL 1962) this method was also abandoned Furthermore the muscle fibers in denervated muscle are known to have electrical properties different from normal muscle fibers (e g NICHOLLS 1956)

In order to elicit a muscle fiber activity in normal muscle a method of chemical activation has been developed It is an old observation described by KUHNE (1859) and others before him and correctly interpreted by *RINGER (1886) that a muscle immersed in calcium free solution becomes* spontaneously active

There is now a vast literature on the mechanisms underlying this spontaneous activity Extensive reviews of this subject have been presented by BRINK BRONK & LARRABEE (1946) BRINK (1954) SHANES (1958) In short the calcium ion stabilizes the cell membrane A lack of calcium makes the cell membrane "leaky and the resting potential unstable When a certain trigger value of the membrane potential is reached that also seems to be dependent on the calcium level a complete depolarisation process is started

The frequency of discharge is dependent upon the amount of calcium removed

In the method worked out an artificial pacemaker was created in the muscle fibers by deprivation of calcium brought about by local injection of a minute quantity of a sodium citrate solution The local application of a citrate solution to a frog muscle preparation has been described by ADRIAN & GELFAN (1933) and to the frog nerve by BRINK *et al* (1946) but has not been previously used as a method for producing fiber activity in the mammalian muscle *in situ*

It will be shown that by this method it is possible to induce an asynchronous activity in individual muscle fibers and consequently the method can be used to define the criteria to be fulfilled by single muscle fiber action potentials

Procedures

It was found to be difficult simply to inject the stimulating solution using an ordinary syringe and then try to find the stimulated fibers with the recording electrode Therefore it was convenient to position the injection cannula and the recording electrode on the same small group of fibers and this was done by electrical stimulation

The short head of the brachial biceps muscle was chosen for these experiments as the fiber direction is known and the innervation zone is principally confined to the proximal and the central part of the muscle (BUCHTHAL *et al* 1955 *a* CHRISTENSEN 1959) By making the injection distally in the muscle the risk of activating terminal nerve fibers ought to be small

To be able to detect action potentials reflexly evoked by activation of sensory structures and those caused by voluntary activation the multielectrode was inserted distally to the innervation zone which was checked by noting the direction of propagation of the voluntary action potentials picked up by the multielectrode (see page 26) It was ensured that the multielectrode was entirely outside the innervation zone by observing about 30 different voluntary action potentials at different sites in the same cross section of the muscle

The injection electrode" (Fig 6) was then inserted some centimeters more distally in the muscle In order to place the stimulating and recording electrodes on the same fibers the procedures of BUCHTHAL *et al* (1955 *a*) were used The potentials evoked were sought with a high stimulating voltage By decreasing the voltage and continously adjusting the position of the two electrodes it could thus finally be ascertained that the two electrodes really were located near the same fibers

In this position the electrodes previously used for stimulation were coupled to one amplifier channel for monitoring the activity and a few microliters of the 0 1 M sodium citrate solution were injected (see page 27)

There could be a delay of up to a few minutes before the spontaneous activity appeared Then a few spike sources within the uptake area started firing Their behaviour was however capricious They could suddenly cease or start to dis

charge violently without any obvious reason The initial positioning of the injection cannula and the multielectrode could only be approximate and once the chemical activity had started the multielectrode had to be re positioned on one of the active spike sources

Action potential recording

The general procedures given on page 24 were followed The 3+11 multielectrode was used The following program could rarely be completed during the short time available due to the capricious behaviour of the action potentials Therefore it was necessary to concentrate on a certain part of the program for each recording so that a complete picture was obtained from the whole sample of recordings

Recording program

1 Ascertaining the needle position so that electrodes 1 and 8 were correctly aligned with the spike source

2 Checking the direction of propagation of the action potentials so as to be sure that the impulse had been initiated near the site of injection

3 Recording the action potentials from electrodes 1 and 8 both with the needle in optimum position and when moving the needle to-and fro

4 Comparisons of the shape of consecutive action potentials both directly on the oscilloscope and indirectly from photographs with at least five superimposed action potentials

5 Recording from all the electrodes in the long electrode row with electrodes 1 and 8 aligned with the spike source

6 Simultaneous study of the bipolar recordings 9–8 and 9–10 while moving the needle to-and fro For the reasons for using these electrode combinations see page 18

Material

Fourteen experiments have been performed in eight young adults without gross signs of neuromuscular disorders Recordings based on the above program have been made from about 500 different spike sources but as mentioned previously only a part of the program has been performed on each

Results and discussion

The type of activity elicited by chemical stimulation

Results

The injection of a minute quantity 10–20 μl of the stimulating solution usually elicited a sparse activity by only a few of the spike sources within the uptake area of the electrode The discharge rate was of the order of

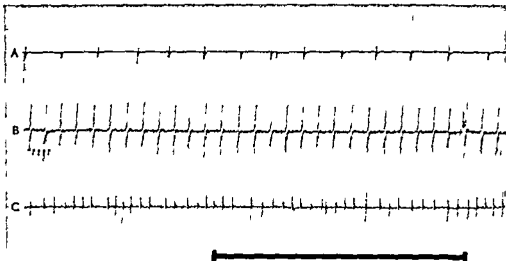


FIG 8 *Ink recordings of chemically elicited activity from single muscle fibers at different repetition rates* Calibration 1 sec

1–5/sec The activity was like that of fibrillation at denervation (Fig 8) Injecting more citrate solution increased the activity so that more spike sources became active and the discharge rate sometimes increased to give a chaotic interference pattern After the injection of larger amounts of citrate a grouping of the spikes occasionally appeared like fasciculations so that 2–4 potentials discharged together (Fig 9 *A*) However there was a considerable variability of an order of magnitude of milliseconds in the mutual time interval so that even the order of the components could vary from discharge to discharge Often one of the components would miss a discharge The variability was usually so gross as to be detectable on the ink recorder with a paper speed 50 mm/sec (Fig 9 *A B C*)

Sometimes there were repetitive discharges in bursts of 2–5 potentials all from the same generator (Fig 9 *D*) as commonly seen in tetany The mutual time interval could vary between 3 5 and 8 0 msec but was usually rather constant between the components and from burst to burst It cannot be settled with certainty if this type of activity was dependent upon the amount of citrate injected or the site of injection

Another type of activity were intense bursts with repetition rates of the action potentials up to 200 per second and a duration of 0 5–3 seconds (Fig 9 *E*) This activity seemed to have been generated by one single source The burst could start and stop either suddenly or gradually like the dive-bomber phenomenon encountered in myotonic dystrophy It seemed as if

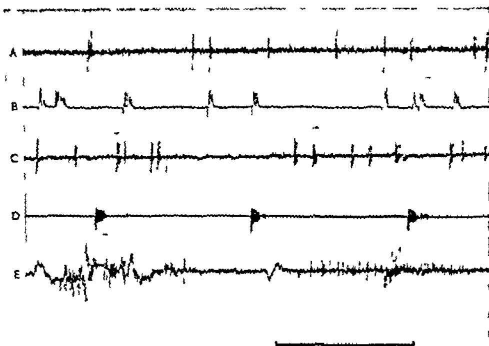


FIG 9 *Ink recordings of action potential groupings (A B and C) repetitive discharges (D) and the dive bomber phenomenon during chemical activation (E)* See page 34 Calibration *A C D* and *E* 1 sec *B* 0 5 sec

these last mentioned types of activity could only be elicited from certain rare sites in the muscle

All the different types of activity could usually be started or intensified by the mechanical stimulation that was obtained by moving the injection needle or tapping with a finger

Discussion

It seems most likely to assume that the regular and quiet fibrillatory activity most commonly encountered actually derived from ordinary (extra fusal) muscle fibers It is however essential to know if the activity really was *single* fiber activity or if more than one fiber was involved in generation The present finding of grouping of the action potentials after the injection of greater amounts of citrate is of special interest in this respect Due to the gross variability in the mutual time intervals and because the citrate was injected in a part of the muscle where motor end plates are sparse it does not seem likely that the activity could have been evoked by stimulation of intramuscular motor nerve fibers However partial synchro-

nization of muscle fibers in calcium deficient solutions was first reported in the frog muscle preparation by ADRIAN & GELFAN (1933) They found the phenomenon also after curarization so excluding nerve stimulation as a causative factor The conditions under which synchronization occurred in those experiments seem to resemble those in the present experiments They found that the synchronization waves only occur at high frequencies the maximum has varied from 90–120 a sec and the minimum has never been less than 60 When the frequency has fallen to this value the discharge breaks up into the usual rapid irregular type though an occasional large wave may appear from time to time" The authors state that the essential conditions for such an occurrence seem to be (*a*) an intense and fairly uniform activity in several fibres, and (*b*) the existence of damaged or permeable regions at neighbouring points in them These would bring the interiors of the fibres into free electrical communication and give a chance to the group to behave as a single unit An active region developing in one fibre close to the injury would tend to activate not only the neighbouring points in the same fibre but also those in the fibres next to it, and thus a rhythmic discharge in one fibre might come to dominate the rhythm of its neighbours" BULBRING HOLMAN & LULLMANN (1956) studied the frog muscle in a calcium deficient solution under the microscope and confirmed the observations of ADRIAN & GELFAN (1933) that at moderate activity the fibers fired asynchronously Further they found that not all neighbouring fibers were active at the same time Intracellular recordings showed that the degree of depolarisation necessary for "triggering" was constant within 5 mV for a certain fiber but differed from the voltage needed in the other fibers of the preparation They also observed partial synchronization phenomena at higher degrees of activity especially in conjunction with mechanical stimulation or after application of histamine

KUFFLER (1944) described the activity of the muscle in calcium free solution as a non-synchronous single fiber activity but, at times synchronized action potentials could appear and these he interpreted as due to a mutual influence of partly depolarised *end plate* regions

TAMAI ABE & GOTO (1961) described a synchronization phenomenon due to direct interaction between adjacent fibers in a frog muscle preparation in a calcium-free solution the synchronization was however no obligate phenomenon, but could happen now and then at the height of the excitability increase

Both in the present and in previous investigations it has thus been found that an action potential grouping with great temporal dispersion and with *loose* binding of the component action potentials may exist. It must, however be emphasized that in all the previous experiments that were described

above the partial synchronization phenomenon was observed with *the whole* muscle immersed in calcium-deficient solution In the present experiments a *small part* of the muscle fiber was made deficient in calcium and this was at least 20 mm away from the recording site The partial synchronization seems to appear only after application of larger amounts of citrate Thus it is most unlikely that synchrony without *any* temporal dispersion and with *fixed* binding of the components—i e equal to within a few microseconds from discharge to discharge—should exist at the low degree of activity obtained after the injection of a minute quantity of citrate The conditions for mutual interaction ought then to be considerably less *The action potentials of the fibrillatory type at low discharge rate seem thus to be generated by single muscle fibers*

The high frequency bursts of activity and the divebomber phenomenon were strikingly similar to the type of activity that has been attributed to intrafusal muscle fibers by BONSETT & ABREU (1963) These authors were able to record this type of activity in advanced cases of muscular dystrophy where the muscle spindles are known to survive until late in the progress of the disease and could obtain histological evidence for their recording using the electromyotome (cf page 15) The intrafusal muscle fibers are also known to become spontaneously active upon calcium deprivation (e g BRUGGENCATE & SCHULTE 1963) The present finding that mechanical stimuli could initiate the activity and that it could be elicited only in certain positions of the injection cannula further supports the above interpretation The question needs however further study before definite conclusions can be drawn

The single fiber action potential recordings

Results

The action potentials recorded at slight activity of the type considered to have been generated by single muscle fibers were smooth biphasic spike potentials (Fig 10) of a shape similar to those recorded from the isolated frog muscle fibers by HAKANSSON (1956 1957 *a*) A third positive phase of low amplitude and long duration was sometimes seen Action potentials of amplitude up to 12 mV were recorded At least 10 action potentials were about 10 mV in amplitude The spike duration varied between 500 and 1000 μsec and the rise time was 80–150 μsec With increasing recording distance the voltage decreased and the rise time increased but the action potential shape was always clean and smooth without irregularities

The action potentials from electrodes 1 and 8 could be made equal in shape and voltage by rotation adjustments of the needle (after correction for the cross talk distortion pages 17 and 75) When moving the needle to-

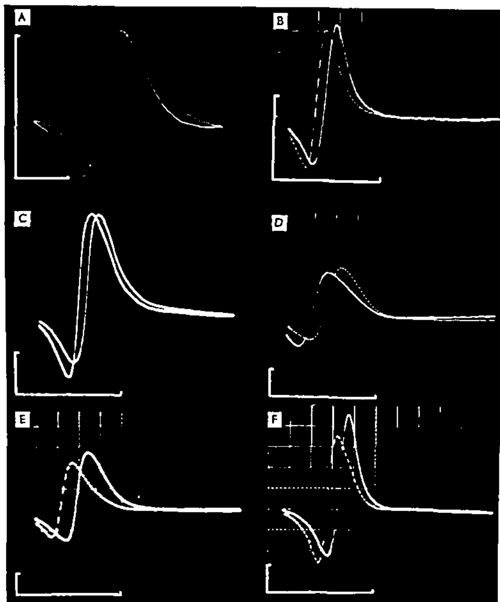


FIG 10 *Examples of chemically elicited action potentials* Single sweep recordings from electrodes 1 and 8 of the 3+11 multielectrode Calibration 2 mV and 500 μsec

and fro the action potentials were always equal and attained maximum voltages for the same needle position provided the electrodes 1 and 8 were correctly aligned with the fiber

From any electrode which was kept in constant position to the fiber the consecutive action potentials were of identical shape (Fig 11) if the discharge

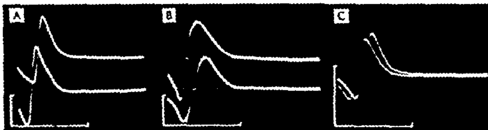


Fig 11 *Superimpositions of consecutive action potentials obtained during chemical activation* Recordings from electrodes 1 and 8 of the 3+11 multielectrode Five discharges are superimposed in Fig *A* eight in Fig *B* and ten in Fig *C* Calibration 2 mV and 500 μsec

rate was low In the repetitive bursts of activity the amplitude was sometimes continuously decreasing within each burst

It was very difficult to obtain suitable recordings from the different electrodes in the long row as this procedure was rather time consuming Therefore it has not been possible to obtain exact quantitative data but the following observations were made There was a steep voltage decrement with increasing distance (cf Fig 34 page 73) Action potentials recorded at the same distance from either side of the fiber were of equal shape and amplitude and were simultaneous The baseline intersection was simultaneous for the action potentials from all electrodes

The bipolar recordings from electrodes 9–8 on one channel and 9–10 on the other could usually be made equal in shape and voltage and simultaneous and this happened for just that needle position when both were of the maximal attainable voltage The maximum voltage was usually $^1/_4$–$^1/_3$ of the voltage of the monopolar action potential from electrode 9 which was also of maximum voltage in the same position By adjusting the needle position either of the bipolar recordings could be made successively smaller until appearing as a nearly straight line Moving the needle further in the same direction made the action potentials increase again but in a reversed form The appearence of a bipolar recording obtained at voluntary contraction is shown in Fig 28 page 62

Discussion of the single muscle fiber criteria

Our knowledge of the single muscle fiber action potentials led off at different distances from the fiber is mainly due to the experiments on isolated frog muscle fibers of Håkansson (1956 1957 *a b*) There are considerable quantitative differences between the human and the frog recordings No direct comparisons can be made because in the present investigation the

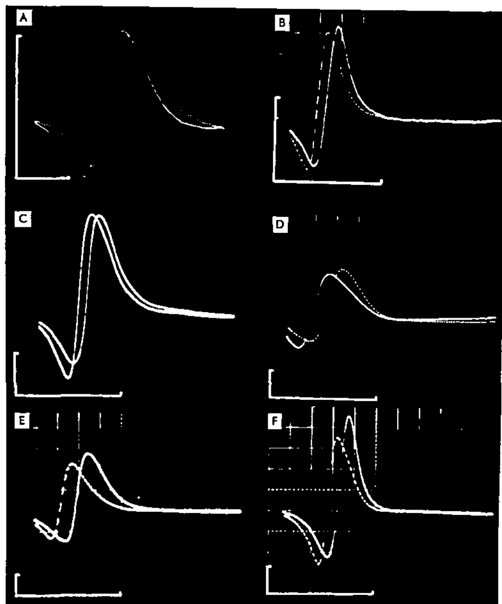


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CHAPTER 3

ACTION POTENTIALS DURING VOLUNTARY CONTRACTION

The conditions for recording a single muscle fiber action potential in the voluntarily activated muscle are that the fiber is close to the electrode and thus gives rise to an action potential of high amplitude and that those other fibers of the motor unit that have coincident action potentials are remote enough from the electrode to make their contribution small Such a potential must fulfil the single muscle fiber criteria (page 40) even if it is not single in the strict sense of the word However are there any conditions under which it is possible that the compound action potentials from two or more adjacent fibers that all give appreciable contributions might fulfil the single muscle fiber criteria? The principal aim of this chapter is to answer this question This has been done by studying *obviously composite* potentials with regard to the criteria in order to gain information concerning what is to be expected from composite potentials of *apparently simple shape* Some quantitative aspects of this question have been studied by means of synthetic composite potentials obtained by a method of adding simple action potentials of known shape amplitude and mutual time interval

The question of the minimum detectable degree of interference from remote fibers on a certain single muscle fiber action potential has also been treated

No systematic study of single muscle fiber action potentials has been found in the literature Usually the possibility of such recordings has not even been taken into account Only BUCHTHAL *et al* (1957 *a*) in discussing the spike of the motor unit potential say that motor unit spikes with low maximum amplitudes derive from sources with a smaller radius than the sources of high amplitude spikes As to the minimum size of the potential source the estimate obtained from volume conduction does not exclude that the source may consist of a single fibre only ADAMS *et al* (1962 page 117) have concluded that all attempts to record single fiber action potentials have failed (cited here on page 6)

Material and procedures

Material

Most recordings have been made in the biceps and the extensor digitorum communis muscles of the arm mainly because these muscles are very easily controlled by the subject After the final settlement of the single fiber criteria about three thousand recordings fulfilling the single muscle fiber criteria have been studied as well as a similar number of recordings where the generator has been two or more muscle fibers This has been made in 105 experiments on 28 subjects mostly young adults but including two children 4 ¹/₂ and 6 ¹/₂ years old The oldest subject was 58 years Seven of the subjects were women None had any signs of neuromuscular disorder

Procedures

With the subject prone and the arm comfortably positioned the muscle was slightly contracted against a resistance No attempts were made to make the contraction strictly isometrical and the force was not measured The slightest possible effort was used to facilitate maintainance of the contraction for a long period and to lessen the effects of fatigue The usual discharge rate of the motor unit was 5–10 per second The room temperature in the earlier experiments was 30–34°C and in the later experiments 22–24°C and at least in the later experiments fairly constant during the course of an experiment (2–3 hours)

In most of the experiments intramuscular temperature measurements have been performed (see page 26) The needle thermo-electrode was in constant position during the course of an experiment because it could not for practical reasons be brought near to the multielectrode each time this was repositioned As there proved to be considerable temperature gradients within the muscle as also shown by BAZETT & MCGLONE (1927) and BUCHTHAL HØNCKE & LINDHARD (1944) the temperature readings could not be considered as a measure of the temperature of the muscle fibers in the region from which the recording was made Therefore the temperature readings in each individual recording are not given They generally fell between 34° and 36°C

The multielectrode was usually inserted about halfway between the end plate zone and the tendon according to the procedures described on page 24 ff A perpendicular position of the electrode row in relation to the muscle fiber was always sought

Simulation of composite potentials

The shape of a composite potential composed of two *simultaneous* single fiber potentials is as clean and smooth as each of the components Only if there is a sufficiently large time interval between the components will an irregularity be seen in the shape What is the minimum detectable time interval? To answer this and similar questions a method was developed where action potentials of known shape amplitude and mutual time interval were electronically added to give a "synthetic composite potential

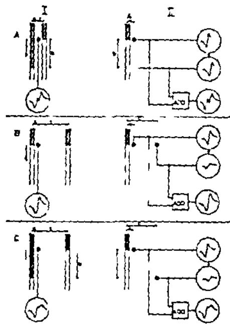


FIG 12 *Simulation of composite potentials* The composite potentials obtained by recording the action potentials from *two* identical fibers with *one* electrode (column I) equal those obtained by adding the action potentials from *one* of the fibers recorded with *two* electrodes (column II) *a* and *c* represent the fiber–electrode distances *b* represents the distance between the depolarisation fronts of the two fibers (column I) and the electrode separation (column II) *A* equidistant fibers with a time interval between their action potentials *B* fibers at different distances from the electrode with simultaneous action potentials *C* fibers at different distances from the electrode with a time interval between their action potentials

Theoretical background In a homogeneous volume conductor the current distribution due to an electromotive force (e m f) is independent of the presence of any other e m f The voltage in a point caused by several e m f will then equal the algebraic sum of the voltage that each e m f should have caused in the absence of the others (HELMHOLTZ 1853) This will also be the case if the volume conductor is inhomogeneous or anisotropic but not in the presence of any non linear elements i e rectifiers or voltage dependent impedances BURKHARDT (1957) remarks that *bei extracellularen Untersuchungen konnen die Nichtlinearitaten durch unphysiologisch hohe Spannungen zwar nachgewiesen werden sie spielen aber normalerweise kaum eine Rolle* Therefore it can be assumed that the results obtained by adding action potentials *outside* the muscle are equal to those obtained when the action potentials are added *within* the muscle BUCHTHAL (1960 1961) has made analyses of compound potentials based upon the same assumption

Addition of action potentials of different voltage shape and mutual time interval can be made as a purely geometrical construction However this is

tedious and can definitely not be used for determining the minimum degree of irregularity that will be detected in an action potential waveform The determinations must be made on the directly observed oscilloscope and on the photographs of the oscilloscopic display in order to include all possible factors Therefore a method was developed for simulation of composite action potentials at the moment of recording The principles are sketched in Fig 12 and can be briefly summarized in the following way The composite potential from *two fibers* recorded with *one electrode* may be simulated by adding the action potentials recorded with *two electrodes* from *one fiber* The interelectrode distance measured perpendicularly to the fiber axis then corresponds to the distance between the simulated and the real fiber The interelectrode distance along the axis of the real fiber corresponds to the distance between the depolarisation fronts of the propagation processes for the real and the simulated fiber

Procedures Both the 3 + 11 and 3 × 4 multielectrodes (Figs 1 and 2 pages 17 and 19) were used the latter mainly when simulating temporal dispersion between the spikes The following refers to the 3 × 4 multielectrode

With electrodes 1 and 9 aligned with a fiber the condition in Fig 12 *A* could be obtained by adding the action potentials from electrodes 1 and 9 the condition in Fig 12 *B* by adding those from 1 and 4 and the condition in Fig 12 *C* by adding those from 1 and 12 The irregular electrode array gave many different similar combinations representing different spatial and temporal intervals For a fiber with propagation velocity 3 5 meter/sec the following time intervals between two equal spikes could be obtained 14 23 27 29 30 41 46 52 55 69 70 73 μsec By using fibers with different propagation velocity other time intervals ranging from 11 to 100 μsec were obtained

Action potentials that fulfilled the single fiber criteria were used

A typical simulation experiment was performed as follows

On channel I of the oscilloscope the simple spike was displayed On channel II the composite potential obtained by adding the output from two electrodes was shown Different time intervals between the two components were selected in random order and it was determined at which time interval the composite character was detected (*a*) without reference to the simple potential and (*b*) when using the simple potential for comparison The determinations were made by direct observations on the oscilloscope screen and on photographs Ten experiments of this type were made

The procedure for using the 3 + 11 multielectrode in simulation experiments is described on page 58

The "double spike

One type of recording that proved to be a most useful tool in the evaluation of the single muscle fiber criteria was the "double spike" (Fig 13) i e two action potentials from the same motor unit each of them fulfilling the single fiber criteria and with a time interval sufficient to prevent interference between them

That the two spikes belonged to the same motor unit was beyond doubt because of their coupling at different discharge rates (cf page 9) The double spikes could not possibly have been repetitive discharges from one single fiber

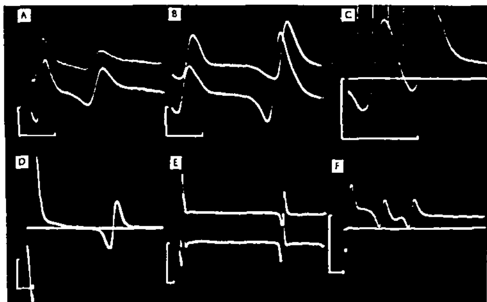


FIG 13 *Double spikes*" and *triple spikes* See page 44 Recordings from electrodes 1 and 8 (*A B F*) or only 8 (*C D F*) of the 3+11 multielectrode Calibration 2 mV and 500 μsec

because the two components usually were of maximum amplitude on different electrodes The double spikes were recorded either from one electrode positioned in such a way that the two components were of equal voltage or each compo nent was recorded from its electrode of maximum amplitude on separate amplifier channels Recording could also be made by bipolar lead-off from the two elec trodes or by adding the signals from the two electrodes by means of the addition device in the amplifier The double spike had a characteristic hollow sound

Results

General remarks on the activity recorded with the multielectrodes

The special design of the multielectrode with the electrodes mounted in an opening at the side of the needle with the small electrode area and with the insulating wall around the electrodes made the electromyogram some what different to that obtained by ordinary concentric needle electrodes The main general characteristics of the recordings will be summarized briefly

At rest there was electrical silence except for the occasional occurrence of end plate noise (BUCHTHAL, 1961) i e miniature end plate potentials when the electrode was positioned in the immediate vicinity of a neuro muscular junction They were purely negative spikes with amplitude up to 200 μV mainly confined to one of the electrodes

There was a striking absence of insertion activity, and this was probably due to the fact that the fibers which were mechanically activated by the tip of the needle were outside the uptake region of the electrodes at the side of the needle Occasionally however a train of impulses, sometimes combined with a dull pain was seen similar to the postulated intrafusal muscle fiber activity found during chemical activation (see page 37)

The action potentials obtained during slight or moderate contraction showed a rich variety of different waveforms the simplest being the fast, smooth spike that fulfilled the single fiber criteria Most other potentials seemed to be composed of two or more such spikes of different voltage and mutual time interval Up to eleven spikes could be discerned in a poly phasic potential In composite potentials of low voltage (less than 0 5 mV) the individual spikes might be difficult to discern appearing mainly as humps or other irregularities of the waveform

The maximum potential voltage obtained with the multielectrode was considerably greater than that with a concentric electrode Maximally 38 mV was obtained (for a composite potential) which is nearly ten times that which has been reported in normal muscle with concentric electrodes (4 2 mV according to BUCHTHAL *et al* 1957 *a*)

At increasing effort the electromyographic activity successively increased up to a chaotic interference pattern The individual action potentials were difficult to discern above a contraction strength of more than about half the maximum because too many motor units were active within the uptake area of the electrode

Tests of the single fiber criteria on composite potentials

In all cases the testing of the criteria was made both by direct inspection of the oscilloscopic display when making the experiment and indirectly from the photographs Judgement of the action potential shape proved to be nearly as accurate when made directly on the oscilloscope as on the photo graphs All voltage and time measurements were made on the photographs

The action potential shape

The single muscle fiber criterion The action potential shape recorded from any of the electrodes is a clean and smooth biphasic spike (Fig 10)

Simulation experiments

The results have been obtained from the experiments described on page 44 When two equal and simultaneous action potentials were added a composite potential of *the same shape* but of double amplitude was obtained

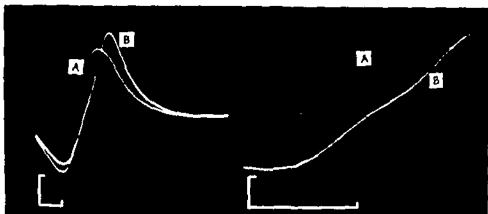


FIG 14 *Simulated composite potentials* Two equal spike potentials of voltage 8 6 mV and rise time 120 μsec added without time interval (*A*) and with time interval 40 μsec (*B*) Recordings from electrodes 1+1 (*A*) and 1+8 (*B*) of the 3+11 multi electrode *Two different sweep speeds are shown Calibration 4 mV and 100 μsec*

At increasing time difference between the action potentials there was a deformation of the rising phase (Fig 14) and when the time interval exceeded the duration of the individual rising phase a notch appeared (Fig 15) It was found that a composite action potential composed of two equal components definitely revealed its character without reference to a simultaneously visible simple one if the time dispersion between the components was more than one third of the individual rise time (as in Fig 14) i e of an order of 30–60 μsec

If the component action potentials were not of the same amplitude the distortions in the wave form altered in character If the trailing component was smaller the notch was closer to the peak if the leading component was smaller then the notch was closer to the trough

The action potentials from electrodes 1 and 8

The single muscle fiber criterion The action potentials recorded from electrodes 1 and 8 are of equal shape when the needle position is adjusted to make both of maximum voltage (Fig 10) They then equal each other also in all needle positions obtained by to-and fro movements

Actual composite potentials

In spite of the small distance (0 2 mm) between the two electrodes the action potentials from the central electrodes in each row were sometimes grossly unequal (Fig 16) This occurred also when the sources of the composite potential were situated close together (30–60 μ) as judged from the sites of maximum amplitudes for the individual components Sometimes the

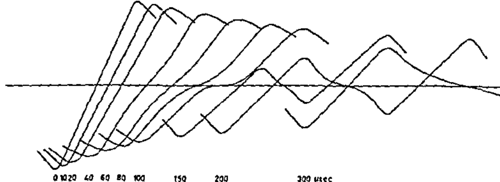


Fig 15 *Shape of composite potentials* composed of two equal components with increasing time interval A graphical construction Rise time of the component action potentials is 100 µsec The numbers below the potentials indicate the time interval in µsec between the components Only the first part of the composite potential is shown for the 300 µsec case

shape on one electrode was rather smooth and clean but on the other clearly composite, even when the two sources were shown to be at a center-to-center distance of 60 µ or less (Fig 16 *B D*)

Simulation experiments

Assume that the composite potentials appear clean and simple on electrode 1 Then one wants to know what is needed for a change to be apparent on electrode 8 The simulation was performed as under *b* on page 44 The minimum time interval obtained was 11 µsec and this gave a very easily recognizable difference between the two potentials It can be concluded that even 5 µsec would be easily detected

The only possible cause for such a difference is a different propagation velocity for the two fibers With one fiber action potential travelling at 3 5 meter/sec the other could then have a velocity of 3 2–3 85 meter/sec without the composite character being detected If the two action potentials were of different voltage and the distortion of the shape thus was in the peak or trough, then the sensitivity in detection of the composite character was less

Consecutive action potentials

The jitter phenomenon

The single muscle fiber criterion Consecutive action potentials are identical in shape (Fig 11)

Actual composite potentials

The action potential shape was not identical from discharge to discharge in *any* of the 3000 composite potential recordings exceeding 0 5 mV in

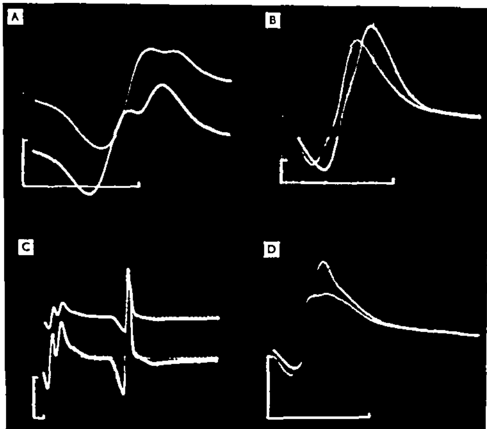


FIG 16 *Composite potentials* differing in shape on electrodes 1 and 8 of the 3+11 multielectrode In *A* and *B* the center to-center distances between the individual spike sources were about 60 μ Calibration 2 mV and 500 μsec

voltage made with the present method nor in any of some 2000 such records made with earlier techniques There was always a variation in the shape usually confined only to a part of the potential (Fig 17) When observed on the oscilloscope the variations were seen as an apparent movement of a part of the display in a restless nervous manner hence called the *jitter phenomenon* (Jitter is adopted from the technical literature where it denotes an instability in an oscilloscopic display due to e g variations in the triggering level or instability in the time base generator)

By the study of composite potentials where two spikes were clearly discernable preferably double spikes (page 44) it was found that the cause of the jitter was a *variability in the mutual time interval between the spikes* and that the individual spikes were of identical shape from discharge to discharge (Fig 18) (A photograph of 58 superimposed consecutive spikes

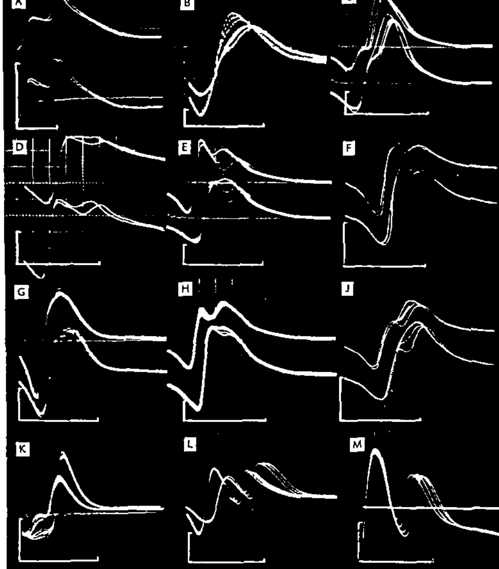


FIG 17 *Action potential jitter* Several consecutive discharges of composite potentials superimposed on each photograph Electrodes 1 and 8 of the 3 + 11 multielectrode In *A C J L M* the center to-center distance between the individual spike sources proved to be 60 μ or less *C F J* have been retouched Calibration 2 mV and 500 μsec

is shown in Fig 19) As the sweep was triggered by the first spike this had a locked position on the oscilloscope at all consecutive discharges, and the time interval differences were reflected as an apparent movement of only the second spike This must not be misinterpreted as there being time varia tions only in the arrival time of the second spike, it is in fact the *difference*

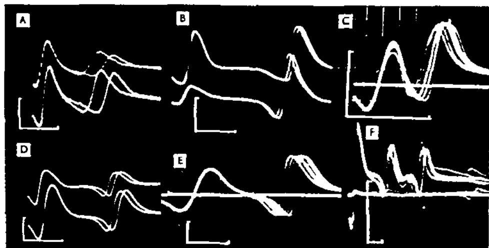


FIG 18 *Jitter in "double spikes"* Superimposed consecutive discharges recorded from electrodes 1 and 8 (*A B D*) or only electrode 8 (*C E F*) of the 3+11 multielectrode *A B C F* are shown as single sweep recordings in Fig 13 Calibration 2 mV and 500 μsec

in arrival time between the two spikes that determines the interspike distance

An analysis of the mutual time interval between the two spikes at consecutive discharges of "double spikes (Figs 20 21) revealed the following characteristics

(*a*) There was an apparently random variability about the mean interval from discharge to discharge i e the jitter phenomenon

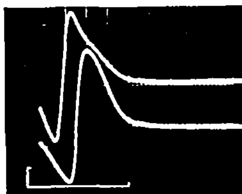


FIG 19 *Superimposition of 58 consecutive action potentials* on one photograph Recordings from electrodes 1 and 8 of the 3+11 multielectrode The shutter was open for 5 6 sec The time interval between consecutive discharges averaged 97 msec with a range of 70 to 180 msec Calibration 2 mV and 500 μsec

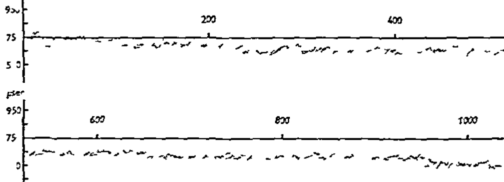


FIG 20 *Inter spike interval in consecutive discharges of double spikes* The two diagrams are a continuous recording of about 1000 discharges over a period of approximately 100 seconds *Abscissae* discharge number *Ordinates* inter-spike interval. There was a gradual decrease in the mean time interval of about 200 μsec during the course of the experiment. The inter-spike interval measured between the baseline intersections of the rising phase of the spikes

(*b*) Sometimes there was a more or less sudden change to an entirely new mean time interval.

(*c*) There were slow variations of the mean time interval It was often observed (as in Fig 21) that the mean time interval gradually increased or decreased (with up to a millisecond) and sometimes one of the spikes then suddenly disappeared

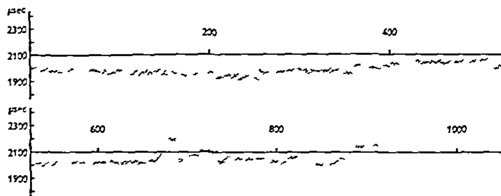


FIG 21 *Inter spike interval in consecutive discharges of double spikes* The two diagrams are a continuous recording of about 850 discharges over a period of approximately 85 seconds *Abscissae* discharge number *Ordinates* inter-spike interval. There are irregular variations in the mean time interval Towards the end of the recording the mean time interval rapidly increased until the second spike suddenly disappeared.

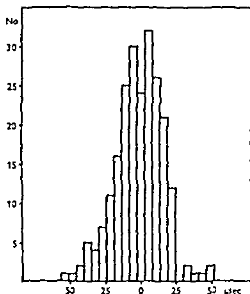


FIG 22 *Distribution about the mean time interval* in consecutive discharges of double spikes S D = 16 6 μsec $N = 223$ During the period of measurements the mean time interval appeared to be constant

In Fig 22 is shown a histogram of the inter spike interval during a period in a recording when the mean time interval was fairly constant However such a constancy was only exceptionally encountered Therefore the standard deviation of the mean time interval could not be used as a measure of the jitter However it was the main purpose of this study to determine the single muscle fiber criteria and in this respect it was not the standard deviation of the time interval that was of interest but the maximum variations during the period where the consecutive action potentials could be compared As it was standard practice to make superimposition photographs of five consecutive action potentials for revealing the jitter the following investigation was performed Consecutive double spikes were recorded for several minutes The material was divided into groups of five consecutive discharges The *maximum and minimum* time difference between the two components i e the range of variation within each series of five was measured The results of measurements on different double spike recordings are given in Table 1 The mean value found in this way will be referred to as the jitter value The jitter value was found roughly to correspond to twice the standard deviation of the mean time interval between the components when the mean was constant The minimum jitter value encountered was 10 6 μsec

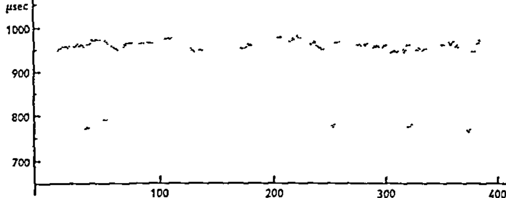


Fig 24 *Inter spike interval in consecutive discharges of the double spike shown in Fig 23 Abscissa* discharge number *Ordinate* inter-spike interval About 350 dis charges during 35 sec The time intervals are grouped about two means (see page 55)

in Table 1 This finding suggests that the variability in the propagation velocity does not contribute appreciably to the jitter but the material is still too small to allow definite conclusions

Ad 3 and 4 No experimental results have been obtained that can clarify the variability of the propagation velocity in the terminal nerve fibers and in the end plate delay and no information can be obtained from the lite-rature

Ad 5 Sometimes a bimodal distribution of the mean time interval be-tween the components of a double spike was encountered Such a case is shown in Figs 23 and 24 70 per cent of the discharges were grouped about a mean time interval of 1 msec and 30 per cent about 0 85 msec Usually as in this case one of the time intervals was more common The phenomenon has been interpreted as being caused by double end plates as discussed on page 67

Comment on the causes of the jitter When recording within the innerva-tion zone of a motor unit the possible effects of a variability in the propaga tion velocity of the muscle fibers ought to be minimized and the jitter in such experiments should mainly be due to factors located within the end plate or in the terminal nerve fibers Considering the highly delicate mechan isms that are involved in neuro-muscular transmission it seems reasonable to assume that *the main part of the jitter phenomenon has been caused by a variability in end plate delay* (Note that it is the variability in the delay in *two* independent end plates that determines the size of the jitter)

The findings under point 5 that might have been caused by double end plates pose the question if part of the jitter could possibly be caused by a

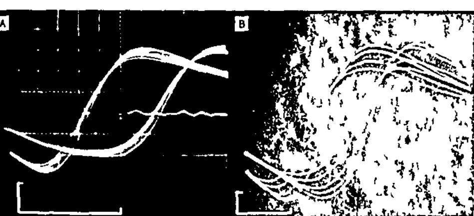


Fig 25 *Movement artefacts* obtained by moving the needle to and fro during exposure Electrodes 1 and 8 of the 3 + 11 multielectrode Calibration 2 mV and 500 μsec

similar mechanism also within each end plate This is further discussed on page 67

The jitter phenomenon and the slow variations in the inter spike intervals need further investigation

The jitter between the action potentials of adjacent fibers Special interest was given to the case when the action potential sources were shown to be close to each other i e when the two component action potentials had their maxima on adjacent electrodes (or even closer), had about equal size and were simultaneous enough to interfere with each other in the trough rising phase or peak This occurred only rarely In the five cases that were studied (Fig 17 *A C J L M*) the jitter does not seem to have been less pronounced than when the component sources were more remote from each other

If the shape of the action potentials from electrodes 1 and 8 differed due to different propagation velocities of the components the jitter was seen in separate parts of the two potentials (Fig 17 *A B C E G H K*)

The 'technical jitter' It must once more be stressed that the jitter in a composite potential appeared as variations in a *part* of the display This is distinct from the variations in shape caused by changes in the electrode position which affect the whole of the action potential (Fig 25) The superimpositions of action potentials from other motor units only produced occasional variations in the shape A 50 cps A C interference from the mains produced an unstable oscilloscopic display due to random fluctuations of the trigger level which also affected the whole of the waveform and was

easily recognizable Any instability in the horizontal deflection circuits of the oscilloscope that might give something resembling the "action potential jitter" can definitely be excluded (see page 29)

Simulation experiments

The actual jitter could not be simulated with the present methods An estimate of the limits within which the shape variations of the action potentials would occur was obtained from the experiments described on page 44, where, instead of comparing consecutive action potentials, as under ordinary conditions two simultaneous potentials were compared (Fig. 14 is an example)

When the two component action potentials were of approximately equal voltage and shape and their mean time interval was about zero the jitter took the form of a tilting of the rising phase about the trough The peak and the trough also underwent small variations in shape The trough was locked in its position by the triggering, which was the reason for the apparent tilting about the trough. With increasing mean time interval the phenomenon became confined to only the negative phase of the potential. When the mean interval exceeded the duration of the rising phase the component action potentials became individually discernible (as in the actual recording in Fig. 17 C)

When one of the components was considerably smaller than the other, the appearance of the jitter was in principle the same but quantitatively less pronounced The case when the jitter seemed to be least apparent was when the component action potentials were nearly simultaneous

It must be mentioned that the jitter under ordinary recording conditions could be considerably modified by the capacitive cross-talk between the electrodes as seen in Fig. 17 F

It was found that a difference of 1 mm between any part of consecutive potentials was detectable both upon direct inspection of the screen and indirectly from the photographs when using the superimposition techniques This corresponds to 2 μsec at a sweep of 20 μsec/cm As the minimum jitter value encountered was 10 μsec it can be concluded that the jitter in a composite action potential where the two components are of about equal amplitude would be detected under all circumstances

To study the situation when the components were of grossly different amplitude the most unfavourable case was chosen namely when the two components were nearly simultaneous The 3 – 11 multielectrode (Fig. 1 page 17) was used A clean smooth action potential with a propagation time of 40 μsec between electrodes 1 and 8 was selected Electrode 3 (at

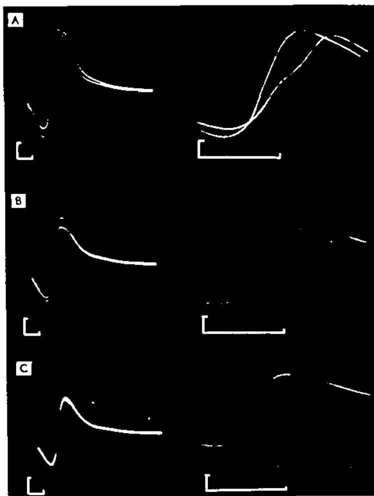


FIG 26 *Simulation of jitter* See page 58 Appearance of the composite potential when two spikes are added without time interval (the clean spikes) and with the main spike leading by 40 μsec In *A* the smaller spike is 50 per cent of the main spike in *B* 10 per cent and in *C* 5 per cent Recording with two different sweep velocities Calibration 2 mV and 100 μsec

one end of the row) was made to overlie the active fiber with the electrode row perpendicular to it The action potentials from electrodes 8 and 1 then equalled each other in shape the former was simultaneous with the action potential from electrode 3 the latter lagged by 40 μsec Both were less than 1 per cent of the amplitude from electrode 8 In one amplifier channel the signals from electrodes 3 and 8 were added in the other those from electrodes 3 and 1 The traces were vertically aligned on the oscilloscope No differences between these two potentials could be detected The interfering

potential was then increased by repeating the procedure with electrode 4 overlying the fiber and adding 4 and 8 on one channel and 4 and 1 on the other and so on

From two experiments of this type it was concluded that the presence of a second fiber with a jitter value of 40 μsec will be detected if the amplitude of its action potential exceeds 5 per cent of the main action potential (Fig 26)

Under actual conditions the jitter appears as a movement of a part of the display Because of the sensitivity of the eye for movement, it is possible that the static simulation experiments underestimate the sensitivity of the procedure for detecting an interference

The action potentials from the different electrodes in the long row

The single muscle fiber criterion The action potentials recorded from the different electrodes in the long row have simultaneous baseline intersections and a steep and symmetrical voltage decrement with increasing distance (see Fig 34)

This criterion was not fulfilled by the actual composite potentials that were studied

Simulation experiments

It must be borne in mind that the voltage decrement only refers to the distances along the long electrode row in the multielectrode and not to the real distances between source and electrode which are unknown Therefore no absolute measured quantity in the decrement curve could be used as a single fiber criterion only the general shape of the curve

To investigate the shape of the curve for the case when two identical fibers with simultaneous propagation were positioned beside each other in relation to the multielectrode one simulation experiment was performed according to Fig 27 A voluntary action potential was chosen fulfilling the single muscle fiber criteria and having a decrement curve similar to that from a chemically activated single fiber and without signs of interference on any electrode Recording was first made according to the procedures described on page 24 with mutual recording distances along the needle of 30 μ so that 22 recording sites were obtained The same procedure was then repeated but the signals from two adjacent electrodes were added at each recording The decrement curve thus obtained equals the one that would have been obtained when recording in an ordinary way from two real fibers lying beside each other as seen from the electrode with a center to-center distance of 50–60 μ (the individual interelectrode distances are not absolutely equal)

In Fig 27 is shown the original voltage decrement curve and that for the

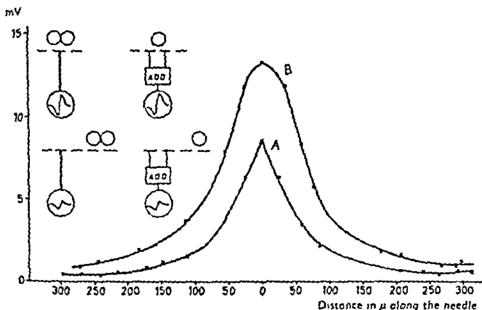


FIG 27 *Voltage decrement curves* See page 60 Spike voltage plotted against distance along the needle Curve *A* is obtained with ordinary recording procedures Curve *B* is obtained by adding the potentials from two adjacent electrodes as shown in the right part of the schematic drawing and it simulates the curve that would have been obtained with two equal fibers positioned beside each other with a center to-center distance of 50–60 μ (the left part of the schematic drawing)

simulated composite potential On either side of the electrode of maximum amplitude (8 mV) the original curve was a continuously decreasing function but the curve for the simulated composite potentials was ʃ shaped and had an inflection point

The diameters of the muscle fibers in the biceps muscle have been measured by BUCHTHAL *et al* (1955 *a*) In an adult muscle from a 62 year-old woman the mean value was 48 μ and the lowest value 25 μ This implies that two fibers of average diameter with simultaneous action potentials lying closely beside each other relative to the needle and at such a distance from the needle as to make each of maximum amplitude 8 mV, would be detected by the shape of the decrement curve

The case with one fiber positioned beside and behind another one in relation to the multielectrode has been constructed from two experimentally obtained decrement curves one with maximum amplitude of 8 mV over electrode 8 and the other with maximum amplitude of 6 mV over electrode 7 The lack of symmetry in the curve was obvious

However when two fibers with simultaneous propagation are positioned exactly behind each other in relation to the multielectrode the decrement

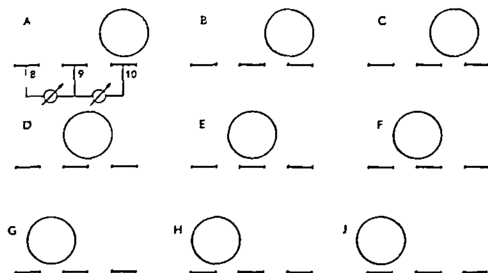


Fig 28 *Recordings fulfilling the bipolar single fiber criterion* The schematic drawing shows the electrode-fiber position for each recording. Note that one of the action potentials is nearly zero in *C* and *G* In *E* the two action potentials equal each other *and* are both at maximum voltage The 3 + 11 multielectrode The dotted traces from electrodes 9–8 Calibration 2 mV and 500 μsec

curve ought to be a continuously decreasing function symmetrical on both sides of the electrode of maximum amplitude This condition is thus not detectable by the decrement curve

The fiber chosen for this experiment must have been relatively close to the needle because the action potential voltage was relatively high At increasing recording distance the decrement curves become flatter (see Fig 34) and the ʃ shape must be more difficult to detect It must also be emphasized that several fibers that are arranged in a round bundle *and* have simultaneous propagation ought to give a decrement curve with a perfectly sharp top If the shape only of the decrement curve is taken into account the present criterion is only a test of symmetry of the action potential source and says nothing about its size

The study of the shape and symmetry of the decrement curves is more conveniently made in the bipolar than in the monopolar recordings (see below)

The bipolar recordings

The single muscle fiber criterion The bipolar recordings 9–8 and 9–10 can be made equal and simultaneous and this occurs in that needle position when they are both maximum The monopolar action potential from electrode 9 is then also maximal By to and fro adjustment of the needle position either of the bipolar potentials can be made successively smaller down to zero The base line intersections for all these action potentials are simultaneous (Fig 28)

Actual composite potentials

As expected this proved to be a most sensitive test for revealing composite potentials The criterion was never fulfilled by the composite potentials It often proved possible to cancel one of the components by appropriate positioning of the multielectrode and thus to discern the other component more accurately This property of the recordings is explained in more detail below Thus the jitter phenomenon was often more clearly visualized in the bipolar recordings especially when the components were nearly synchronous

Simulation experiments

The bipolar recordings from a two-fiber generator were difficult to simulate However from the experimentally obtained monopolar decrement curves in Fig 27 the *bipolar decrement curves* could be geometrically constructed and are shown in Fig 29 In *A* (the single fiber generator) the bipolar recordings are at their maximum amplitude in the same position of the multielectrode and the bipolar action potentials are then of equal voltage In *B* is shown the bipolar decrement curves for the simulated case of two fibers beside each other with a center to-center distance of 50–60 μ

mV

A

B

300 250 200 150 100 50 0 50 100 150 200 250 300

Distance in μ along the needle

FIG 29 *Bipolar voltage decrement curves* constructed from the monopolar curves in Fig 27 *A* shows the curves obtained from a single fiber with electrode combinations 9–8 (dotted lines) and 9–10 (full lines) The curves have coincident maxima *B* shows the curves for the simulated case with two fibers beside each other The maxima of the two curves in *B* are separated by 60 μ All distances have been counted from the electrode that is common for the two bipolar pairs (electrode 9)

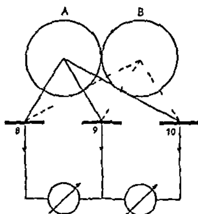


FIG 30 *Method of cancelling fiber action potentials by bipolar recordings* The idealized case with two 60 μ fibers closely beside each other Bipolar recording electrodes with a center to-center distance of 60 μ The action potential from fiber *A* is cancelled by bipolar recording from electrodes 9–8 and from fiber *B* by bipolar recording from electrodes 9–10 Thus electrodes 9–8 record the action potential from fiber *B* and electrodes 9–10 the action potential from fiber *A* exclusively

The two bipolar recordings have their maxima separated Little doubt exists that the composite character should be revealed in this case

If the multielectrode should be positioned in such a way in relation to two adjacent fibers as shown in Fig 30 the right bipolar recording should be dominated by the left fiber and the left bipolar recording by the right fiber Then even a slight asynchrony in the components of the order of 2 μsec should be detected by the non simultaneity of the potentials Triggering the sweep from one of the bipolar potentials should make the jitter appear as to and fro displacements of the other bipolar potential

The case when two fibers are positioned strictly behind each other is not detectable as any asymmetry or separation of the maxima

The bipolar recordings as used here are useful as tests of the symmetry of the action potential source but give no information as to its size The sensitivity in the detection of an unsymmetry decreases with increasing recording distance

Recordings fulfilling the single muscle fiber criteria

Action potentials that fulfilled the criteria for single muscle fiber potentials in every detail were found frequently in the voluntarily activated muscle However it was a common experience to find interference from other fibers of the motor unit when recording from the outer electrodes in the long electrode row It was however usually not difficult to construct the individual decrement curves for both the fiber overlying the central electrode and the interfering fiber so that the voltage of the action potential from the interfering fiber could be calculated for the central electrode An unsymmetrical decrement curve when the cause is thus analyzable, can still be compatible with a single fiber recording However it sometimes occurred that the decrement curve was unsymmetrical for other reasons When for instance an action potential was found immediately after the needle had pierced the fascia felt as a tough resistance a number of electrodes could be within and the others outside the fascia making recording conditions different in the two parts of the electrode row

It is also possible that there may be inhomogeneities within the bulk of the muscle that could influence the action potential decrement in a similar way This question will be further discussed in Chapter 4

No recording was accepted if the decrement was not strictly symmetrical over the three central electrodes as checked with the bipolar recordings

There were considerable differences in different muscles as to the frequency of occurrence of simple potentials In the brachial biceps or frontalis muscles potentials fulfilling the criteria were easily found usually at each

insertion of the needle, but in the tibialis anterior and gastrocnemius muscles such recordings were only rarely encountered In the extensor digitorum muscle of the arm and the opponens pollicis muscle the recordings were often polyphasic with the individual spikes clearly discernable There were also considerable differences between the same muscle in different subjects, potentials fulfilling the criteria being more easily found in the gross muscles of men than in the thin muscles of women or children There were also differences in this respect between subjects having muscles of approximately the same mass

The shape of the action potentials will be reported and discussed in Chapter 4 in conjunction with the distortion factors that have been introduced by the method

Discussion

The jitter phenomenon

The jitter phenomenon does not seem to have been described in earlier literature In human electromyography, when using ordinary concentric needle electrodes, it is clearly seen (page 83) but tends to be obscured by the instability in the wave form that is caused by movements of the electrode in relation to the fibers Such movements are difficult to avoid when the electrode is on the bevel surface of the needle tip The jitter is difficult to notice when using a free running sweep on the oscilloscope, as is usually the practice

The jitter phenomenon has not been described in animal nerve muscle preparations

It has been postulated (page 56) that the action potential jitter is mainly due to a variability in the end plate delay i e the time interval between the arrival of a nerve impulse to the nerve-ending and the start of the propagated depolarisation in the muscle fiber In the frog nerve muscle preparation kept at 20 C the end plate delay is 1 1–1 3 msec It increases at partial curarization and at fatigue (KUFFLER 1944) In the human end plate at 37 C the end plate delay seems to be unknown but is probably shorter

There are reasons to believe that the jitter is largely influenced by random factors The jitter-value corresponded to roughly two times the standard deviation (S D) it is to be expected that 2 out of 6 consecutive intervals should fall outside the range ±1 S D which is in good accordance with the experimental results (Table 1)

With the assumption that the jitter is the variability in the end plate delay of *two* end plates and that the variability for each end plate is equal the

individual variability expressed as the standard deviation is $1/\sqrt{2}$ of the variability of the inter spike interval and is hence of the order of magnitude of 5–20 μsec

The occurrence of a bimodal distribution of the inter spike distances in consecutive double spikes in the case illustrated in Figs 23 24, affords an interesting interpretation About 2 per cent of the muscle fibers in human muscle are known to have double end plates innervated by the same neurone (COERS & WOOLF, 1957) If the impulse in the muscle fiber should start alternately from the two end plates there ought to be a bimodal distribution of the difference in the arrival time of the action potentials Now it is reasonable to ask if part of the apparently random variations about the mean in fibers with a single end plate could be due to a similar mechanism The end plate is not a plate but has a highly complex branching pattern and it can be imagined that initiation of the propagated impulse from different parts of this structure at different occasions could be partially responsible for the jitter However the mechanism involved in neuro muscular transmission i e setting free of the acetylcholine at the arrival of the nerve impulse the depolarisation process of the end plate the initia tion of the propagated impulse and the inactivation of acetylcholine and recovery, is so complex that some variability in neuromuscular transmission time is highly probable

It seems that not only the jitter phenomenon but also the slower varia tions in the inter spike interval that have been found (page 51) could be of interest for the study of end plate physiology and pathophysiology In myasthenia gravis where neuromuscular transmission is directly affected the conditions ought to be different from the normal as in certain myo pathies where the end plates are known to be malformed and spread over great lengths of the fibers and also in denervation and subsequent regeneration by collateral sprouting from other axons Also investigations of the jitter under influence of neuromuscular active drugs ought to be of interest

The single fiber criteria

The possibility that two fibers at about the same distance from the electrode should have been the generator of a recording that fulfils the single muscle fiber criteria seems to be most unlikely The most important proof for this statement is the presence of the jitter phenomenon that ought to reveal such a composite potential under all circumstances But also if the jitter is left out of account the remaining criteria seem to offer good evidence in support of this statement

What conditions are necessary for two adjacent fibers to generate a com

pound action potential that fulfils the criteria? As to the spatial arrangement it is unlikely that they could be positioned beside each other because this should reveal itself as a blunt appearence of the decrement curve (page 60) together with a separation of the sites of maximum amplitude for the two bipolar recordings (page 63) (at least if the fibers are so close to the electrode as to make their individual action potential 8 mV or more) If one fiber should be behind and beside the other there should also be an asymmetry in the decrement curve and in the bipolar recordings (page 61) The two fibers must then be positioned behind each other in relation to the multielectrode (page 61) to escape detection

In order that the composite action potential should be perfectly smooth and clean in shape it is necessary that the arrival time of the two component action potentials be simultaneous to within 30–60 μsec (page 47) If the two fibers should have exactly the same site of initiation of the impulse i e the same location of the end plates and the recording is made at a distance of 35 mm from the site and assuming a propagation velocity of 3.5 meter/sec for one fiber, then the other must have a propagation velocity of 3 5±0 01–0 02 meter/sec to mask the composite character In other words the propagation velocities must be equal to within 0 3–0 6 per cent If the two fibers should have different locations of their end plates there must be a corresponding difference in the propagation velocities to within 0 3–0 6 per cent to make them simultaneous at the site of recording This ought to happen only very rarely, provided that there is no synchronization between the fibers Such a synchronization is assumed by BUCHTHAL *et al* (1957 *a*) to explain the smooth clean shape of the subunit - in spite of its being generated by up to 30 fibers of different nd with different sites of innervation No evidence for any tion has been found in the present investigation (unec of adjacent fibers, page 47 and jitter between a not consistent with a synchronization process) discussed in Chapter 6 on page 87

Thus it can be concluded that *two adjacent* ... *give a recording that fulfils the single muscle* fibers in a bundle can definitely not give such

It was stated in the introduction to this c interference from the rest of the motor un must l fiber The possibility of detecting an depends on the jitter value the mean t potentials of the main and the interfering fibers in relation to the electrode It was under the unfavorable conditions when

behind the main fiber and the mean time interval between their spikes was nearly zero that the voltage of the interfering spike potential must exceed 5 per cent of that of the main spike in order to be detected provided the jitter value exceeded 40 μsec *Assuming* the same conditions a reasonable estimate would be that if the jitter value should be 20 μsec the interfering voltage must be 10 per cent to be detectable and with a minimum jitter-value of 10 μsec it must be 15 per cent Since most jitter values were above 20 μsec an interference from one spike with amplitude 10 per cent of the main spike should be detected whereas interference from a spike smaller than 5 per cent may easily be missed

However it is possible that *several* fiber potentials each of low amplitude might summate to give an interfering composite potential Even here the jitter phenomenon ought to be visible and there are no reasons why the jitter should be less pronounced in such a case than with an interfering single spike

Concluding remarks on the single muscle fiber criteria

The investigation has shown that *spike potentials that were of identical shape at consecutive discharges were single muscle fiber potentials* This must be true when using any type of recording system provided the time resolution is sufficient to permit the detection of a variability in a part of the wave form of the order of 10 μsec According to the experience gained in the present investigation no criterion besides absence of jitter is needed This implies that all the electrodes in the multielectrode are by no means necessary for the selection of single muscle fiber potentials as they can be as efficiently selected with only one of them However it is necessary that the electrode position in relation to the fiber be kept fixed in order that no movement artefacts obscure the jitter

PARAMETERS OF THE SINGLE MUSCLE FIBER ACTION POTENTIALS WITH PARTICULAR REGARD TO THE DISTORTIONS

The extracellular action potential represents the potential variations in a point outside the muscle fiber caused by the electrical events over the muscle fiber membrane. The shape of the action potential is determined by the properties of the muscle fiber, the properties of the volume conductor surrounding the fiber and by the distance between the fiber and the electrode. Furthermore, some distortion factors are inevitably introduced by the recording system. Apart from these physical distortions there are also "statistical distortions" affecting, for example, the size distribution.

In this chapter the single muscle fiber action potentials from the material in Chapter 3 are discussed with particular regard to the distortions. The aim is, however, more to indicate the sources of distortion rather than to make a quantitative evaluation of them. An extensive review of this subject is that of BURKHARDT (1957).

Results

The single muscle fiber action potentials

The single muscle fiber action potentials obtained during voluntary contraction had similar shape to those obtained during chemical activation (Fig. 10, page 38). They had a smooth, biphasic spike-component, often followed by terminal phases of low amplitude and long duration (Fig. 31). The positive phase of the spike was usually of 10–20 per cent lesser voltage than the negative phase. Occasionally the positive phase could be absent or small (Fig. 31 C), considered by BUCHTHAL (1961, 1962) to occur when recording near the end plate.

About 300 action potentials from 28 of the experiments made in the brachial biceps muscle of 15 young men and 4 young women have been measured. All action potentials have been obtained after adjustment of the electrode position for optimum spike voltage. The spike voltage, rise time

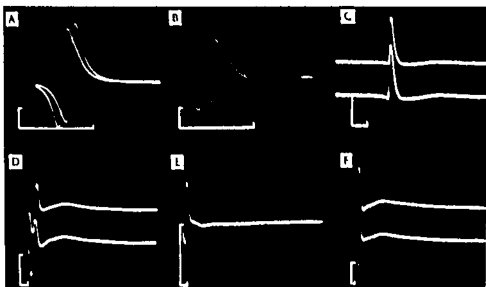


FIG. 31. *Examples of single muscle fiber action potentials.* The 3+11 multielectrode. *A, B, D, E* from electrodes 1 and 8, *F* from electrode 8. Both potentials in *C* from electrode 8 and probably recorded near the end plate, the upper with pass band 60–35 000 cps and the lower with 5–35 000 cps. The potentials in *D* with pass band 5–35 000 cps. The other potentials with pass band 60–35 000 cps. Calibration: 2 mV and 500 μsec.

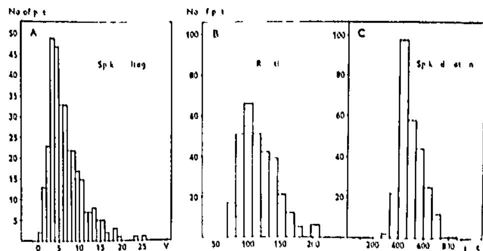


FIG. 3[illegible]. *Spike voltage, rise time and spike duration* for single muscle fiber action potentials (measured according to Fig. 7). *A. Spike voltage*, median value 5.6 mV, range 0.7–[illegible]5.2 mV (n = 30[illegible]). *B. Rise time*, median value 112 μsec, range 67–[illegible]00 μsec (n = 310). *C. Spike duration*, median value 470 μsec, range [illegible]65–800 μsec (n = 26[illegible]).

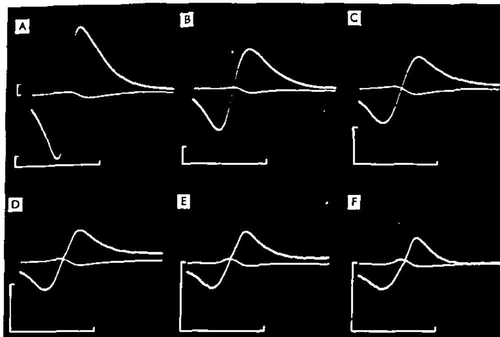


FIG 33 *Recordings obtained at different distances from a fiber* Electrodes 8–3 (*A–F*) of the 3+11 multielectrode with electrodes 1 and 8 aligned with the fiber The bipolar lead-off 7–9 is used as a reference in each recording There is a considerable cross talk component from electrodes 1 and 8 in *D E F* Calibration 2 mV and 500 μsec

and spike duration are shown in Fig 32 The three distribution curves show a positive skewness The median value for the voltage was 5 6 mV for the rise time 112 μsec and for the duration 470 μsec

Thirteen action potentials (4 2 per cent) were above 15 mV, with maximum value 25 2 mV Ten of these recordings were obtained from male subjects with markedly well developed muscles

Five voltage decrement curves (i e the spike voltage plotted against the *distance along the needle*) are shown in Fig 34 The shape of the action potentials in such an experiment is shown in Fig 33

Discussion

The distortion factors

The insulating wall around the electrodes

The action potentials were not recorded in an unlimited volume conductor but at the boundary of a volume conductor and an insulator namely the surface

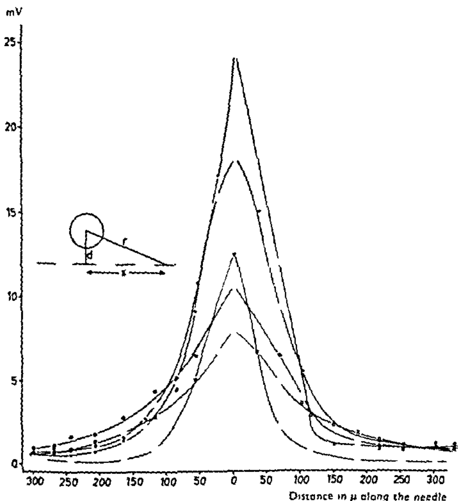


FIG 34 *Voltage decrement curves* Spike voltage plotted against the distance along the electrode array of the 3 + 11 multielectrode (*x* in the inset) The fiber–needle distance (*d*) and the fiber–electrode distance (*r*) are not known The plateaux in the outer parts of some curves are to be attributed to the cross talk component (page 75) Distance "0" corresponds to electrode 8

of the epoxy resin mould in which the electrodes were embedded (Fig 1 page 17) By means of the method of electrical images (see e g JEANS 1933 page 185 STRATTON 1941 page 193 WEBER 1950 page 215) it can be calculated that the effect of introducing an infinitely large insulating boundary in the plane of the electrodes here called the wall effect will be to increase any voltage by a *factor of two* in comparison to that which would have been obtained in an unlimited volume conductor Thus the action potential shape is not distorted by the presence of the insulator but the trough to-peak voltage is doubled

(This result has been confirmed in an electrolytic tank muscle fiber analog where it was also found that the insulator surrounding an electrode must be of

at least twice the diameter of the muscle fiber to exert approximately the full wall effect EKSTEDT & STÅLBERG unpublished)

The presence of the 14 metallic electrodes in the insulator might modify the wall effect and introduce some form of distortion that cannot be determined at present

The metallic needle

The electrodes in the 3+11 multielectrode are somewhat unsymmetrically mounted in the opening of the needle (Fig 1) Electrode 1 is only 70 μ away from one border of the opening while electrode 8 is 270 μ away In spite of this there seems to be no other inequality between the action potentials from electrodes 1 and 8 (when these are aligned with the fiber) than that explained by the cross talk (see below) neither when the impulse passes in the direction from electrode 1 to 8 nor when it passes in the reverse direction

Thus it is reasonable to assume that the metallic needle is too remote from the electrodes to introduce any appreciable distortion

The electrode area

If the electrode is not a point but has a certain area it records the mean voltage distributed over its surface (HÅKANSSON 1956)

Besides this there is another factor that might be of some importance but does not seem to have been taken into account in the literature The platinum surface is polarizable (BUCHTHAL *et al* 1954*a* BURKHARDT 1957) Thus it is a good conductor for high frequency currents but a poor conductor for low frequency currents Therefore it is possible that the high frequency components of an action potential are decreased more by the area of the electrode due to electrical shunting of the currents in the volume conductor than the low frequency components It is even possible that the low frequency components are enhanced, the polarized electrode surface exerting a wall effect (page 72) (This has been shown to occur for polarized electrodes in the electrolytic tank muscle fiber analog EKSTEDT & STALBERG unpublished) However no quantitative estimate of this effect can be made at present

The electrode polarization also causes a low frequency *attenuation* due to the voltage division between the polarized electrode surface and the amplifier input impedance (see e g BURKHARDT 1957) This effect is responsible for the limitation of the low frequency response of the electrode–amplifier system at 1 cps (See page 29)

A rough estimate of the influence of the electrode area is obtainable from the experiment shown in Fig 35 With electrodes 1 and 8 of the 3+11 multielectrode aligned with a fiber the effect of increasing the extent of the recording surface *along* the fiber was studied by connecting electrodes 1 and 8 (Fig 35 *A*) This increased the rise time with an amount equal to the propagation time between electrodes 1 and 8 but the voltage of the potential was only little affected When connecting seven electrodes transversely to the fiber (5–11) (Fig 35 *B*) the rise time was mainly unaffected but the amplitude decreased It may be permissible to draw the analogy that the extent of the 30 μ electrode *along* the fiber has increased the rise time with an amount corresponding to not more than the propagation time across the electrode With a propagation velocity of 3 5 m/sec

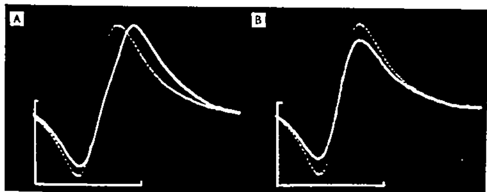


FIG 35 *The influence of the electrode area on the action potential shape* The 3 + 11 multielectrode with a fiber aligned with electrodes 1 and 8 The dotted action potentials in *A* and *B* (voltage 3 6 mV) obtained from electrode 8 The other potential in *A* has been obtained by connecting electrodes 1 and 8 and thus increasing the extent of th recording surface *along* the fiber The rise time is increased by an amount equal to the propagation time (80 μsec) over the combined recording surface but the voltage is not affected very much In *B* an increase of the transverse extent of the recording surface has been obtained by connecting all electrodes from 5 to 11 (370 μ) Th* rise time is hardly affected but the voltage decreases Calibration 2 mV and 500 μsec

this should be about 10 μsec The *transverse extent* of the electrode ought to decrease the potential voltage but the amount of decrease must depend on the fiber diameter in relation to the electrode diameter and on the distance between fiber and electrode which are unknown in any individual case Therefore any quantitative estimate of the voltage decrease introduced by the 30 μ electrode cannot be made at present

The cross talk between the electrode leads

It has already been pointed out in Chapter 1 (page 17) that there was a capacitive cross talk between the electrode leads Some examples of the ap pearance of the cross talk under different conditions are shown in Fig 36 When recording from the outer electrodes in the 3 + 11 multielectrode the cross talked signal could often exceed the amplitude of the volume conducted action potential The plateaux in the outer parts of the decrement curves in Fig 34 are due to the cross talked signals which were usually between 3 and 5 per cent of the amplitude of the largest action potential

The cross talk could be reduced by the use of electrode 3 or 13 as reference but this has rarely been used in this investigation as it made the procedures too tedious (page 20)

Mechanical effects of the needle

The needle multielectrode has approximately 100 times the cross sectional area of a single muscle fiber A great many fibers must be crushed by the needle It has already been stressed (page 16) that when the needle tip was blunt or was

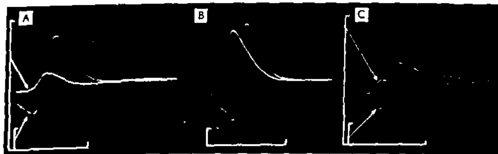


FIG 36 *The cross talk distortion* *A* and *C* made with the 3+11 multielectrode The fiber passes perpendicularly to the needle

In *A* electrode 3 is aligned with the fiber (the larger spike is from electrode 3) and recording is made from electrode 13 (small spike) The latter must be caused almost entirely by capacitive cross talk from electrode 3 since it is equal to the potentials from electrodes 12 and 11 (not shown)

In *C* the fiber is aligned with electrodes 1 and 8 From both of these there is a capacitive cross talk to electrode 3 (the complex small potential) The large potential is from electrode 1 Propagation was in the direction from 8 to 1

B is obtained from electrodes 2 (first potential) and 10 with the fiber aligned with these electrodes of the 3×4 multielectrode The deformation of the initial positive phase in the second spike is caused by cross talk Calibration 2 mV and 500 μsec

bent it was very difficult to obtain action potentials of high amplitude presumably because the fibers lying close to the electrode were damaged The appearance of the action potential from a *partially* damaged fiber cannot be decided at present

Serious consideration must be given to the question of what happens when a great many fibers are crushed in the vicinity of the fiber from which recording is being made It is reasonable to assume that the intracellular fluid rich in potassium ions pressed out from the damaged fibers changes the composition of the extracellular fluid This might alter the action potential either by affecting the membrane potential or by altering the volume conductor in which the action currents are distributed No satisfactory answer can however be obtained at present

The muscle as a volume conductor

This subject has been treated by BURKHARDT (1957)

The action currents from an isolated single muscle fiber immersed in Ringer solution (e g HÅKANSSON 1956 1957 *a* *b*) are distributed in a volume conductor that is homogeneous (all parts of it having the same conductivity) isotropic (the conductivity being the same in all directions) and does not contain any non linear elements (i e elements that do not obey Ohms law) Furthermore the conductivity is purely resistive

The action currents from a muscle fiber *in situ* are distributed in a volume conductor that is inhomogeneous anisotropic and contains non linear elements

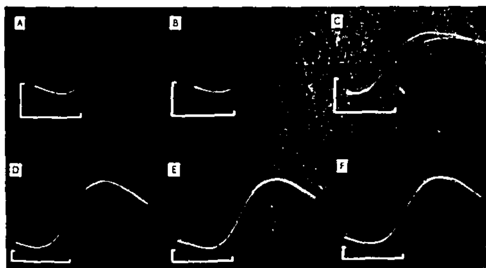


Fig 37 *The effects of limiting the pass band of the amplifiers* Recordings from electrode 8 of the 3 + 11 multielectrode Different potentials in the two rows The larger potentials in all recordings obtained with a pass band for electrodes and amplifiers together of 1–35 000 cps Pass band for the smaller potential in *A* 1–20 000 cps *B* 1–12 000 cps *C* 1–9 000 cps *D* 5–35 000 cps *E* 60–35 000 cps *F* 140–35 000 cps Calibration 2 mV and 100 μsec

Inhomogeneity The electrical properties of the muscle fibers differ from those of the extracellular space (BUCHTHAL *et al* 1955 *b*) and the muscle contains fibrous tissue fat nerves and blood vessels The smooth clean shape of the action potentials from the 11 electrodes and the symmetry of the decrement curves (Fig 34) suggest that the degree of distortion due to inhomogeneity is as a rule small

Anisotropy In the direction of the fiber axis the conductivity is mainly resistive while in the transverse direction both resistive and reactive (caused by capacitors) (BURKHARDT 1957) What effect this may have on the recordings cannot be decided at present

Non linear elements See page 43 where the opinion of BURKHARDT (1957) was quoted that the non linear properties of the muscle are normally of minor importance

The amplifiers

The distortions of the shape of the spike when the pass band of the recording system is limited are shown in Fig 37 Some examples of the influence of the low frequency response on the terminal phases are shown in Fig 31 It seems that the pass band 5–35 000 cps (for the electrode and amplifier together) gives a reasonably faithful reproduction of the action potential waveform However a high pass filter of 60 cps has usually been used for all recordings (unless other wise indicated) and this must have decreased the amplitude of the positive phase and reduced the terminal phases

It can be calculated from the rise time of the electrode and amplifier (12 μsec page 29) that an original rise time of 70 μsec increases to 71 2 μsec (i e an increase of 1 5 per cent) upon recording (calculated by vector addition of the two rise times see TERMAN & PETTIT 1952 page 259)

Summary of the distorting factors in the present methods

1 The insulating wall around the electrodes increases the action potential voltage by a factor of up to two without any other effect on the action potential shape

2 The metallic needle shaft does not distort the potentials appreciably

3 The electrode area of 30 μ increases the rise time by approximately the propagation time across the electrode (10 μsec for a fiber of velocity 3 5 meter/sec) and decreases the voltage, presumably only to a minor degree The exact influence of the electrode area is not yet definitely established

4 There is a capacitive cross talk between the electrode leads that distorts the potentials The cross-talked signal may even exceed in amplitude the volume conducted action potential

5 The needle may damage the muscle fiber under study By crushing adjacent fibers the natural environment of the fiber used for recording may also be altered The importance of these factors cannot be settled at present

6 Inhomogeneity anisotropy, non linearity and the presence of reactive components in the muscle may affect the current distribution in the muscle

7 A limited frequency response of the recording apparatus distorts the recordings By the use of a high pass filter of 60 cps the positive phase and the terminal phases are decreased The high frequency response of 35,000 cps increases a rise time of 70 msec by 1 5 per cent

If the action potentials are used only as time markings to indicate *when* a certain event occurred the distortion may be unimportant This is, for example the case when studying the time variations between the com ponents of a composite potential (the jitter phenomenon page 48) or in the determination of the propagation velocity (EKSTEDT & STALBERG 1963) Determination of the direction of the propagation for delineating the end plate zone (page 32) can be made with highly distorted action potentials A distortion is of little importance when using the action potentials to locate active fibers

However if the action potential waveform shall be used for drawing conclusions about the properties of the muscle fiber then the first step must be to determine the distortions introduced by the recording methods in order to be able to deduce the real potential variations that should occur if the recording electrode had been absent

Parameters of the single muscle fiber action potentials

The action potentials from an isolated frog muscle fiber immersed in a large volume of Ringer solution was maximally 2 mV when recording just outside the fiber (HAKANSSON, 1957 *a*) BUCHTHAL & STEN KNUDSEN (1959) recorded extracellular action potentials with a glass capillary electrode close to the surface of deep frog muscle fibers *in situ* and found the action potential voltage to be 6–7 mV The number of measurements are not given The larger action potential voltage obtained when recording from a fiber in situ than when immersed in Ringer solution was explained as an effect of the lower external conductivity in situ

The maximum value of 25 mV found in this investigation may be astonishing However due to the 'wall effect' the voltage has been increased by a factor of two and ought in fact to have been 12 5 mV if recorded in an unlimited volume conductor Action potentials of such high amplitude were only exceptionally encountered and must have occurred with the electrode in immediate contact with a fiber of very large diameter It is not likely that the high voltage recordings were intracellular recordings because such a recording ought to be mainly a positive spike and the high voltage potentials had essentially the same shape as the low voltage potentials 94 per cent of the recordings were below 15 mV (i e 7 5 mV when correcting for the 'wall effect') and this seems to be in good accordance with the findings of BUCHTHAL & STEN KNUDSEN (1959)

The spike potentials recorded in human muscle by BUCHTHAL *et al* (1957 *a*) using a multielectrode were up to 10 mV in amplitude and these appear to be the largest muscle action potentials so far reported

There is a considerable discrepancy between the voltage distribution in this study and that reported for *motor unit potentials* recorded after random insertion of concentric needle electrodes by BUCHTHAL *et al* (1954 *a*) In that study of 1268 motor unit potentials about 25 (2 per cent) were above 700 μV and none seemed to have been above 1 mV In the present investigation two spikes out of 300 were below 1 mV while none were below 700 μV A part of the discrepancy can be explained by the reduced distortion introduced by the present electrodes due to their smaller diameter (see Chapter 5) The remaining difference may however be explained in the following way the action potentials *selected* for study in the present investigation were of a type that were only quite exceptionally encountered when recording by *randomly* inserted electrodes In the present study an amplifier sensitivity of 100 μV/mm on the oscilloscope screen was used in seeking for the action potentials and therefore those below 500 μV were neglected No doubt this has introduced a bias in the sampling of the action

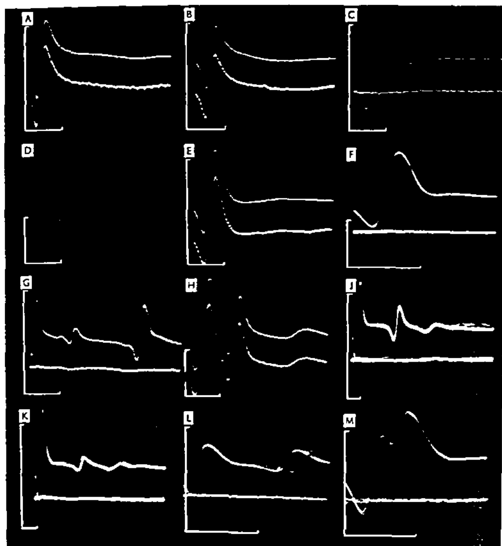


FIG 38 *Recordings with concentric needle electrodes* In all recordings the upper potentials are led off between the central electrode and the shaft of the needle In *A B E H* the lower potentials are led off between the central electrode and a subcutaneous reference In *F* about 15 potentials are superimposed in *J* about 12 in *K* about 8 in *L* about 7 and in *M* about 8 *D* has been retouched Calibration 2 mV and 500 μsec.

difficult to obtain isolated spike components with this electrode than with the multielectrode In the composite potentials above 0 5 mV the *jitter phenomenon* between the component spikes was invariably seen and was of essentially the same appearance and size as when recording with the multi electrodes (Fig 38 *J K L M*) *The individual spikes were of identical shape*

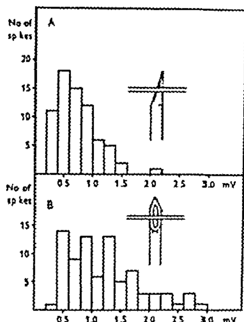


FIG 39 *Single muscle fiber spikes from the concentric needle electrodes* The insets show the electrode position in relation to the fiber In each case the action potentials have been adjusted to maximum voltage by to-and fro movements of the needle
A Arithmetic mean 0 7 mV Median value 0 7 mV Range 0 2–2 1 mV $N = 70$
B Arithmetic mean 1 2 mV Median value 1 2 mV Range 0 2–2 9 mV $N = 83$

at consecutive discharges (Fig 38 *F*) Their shape was similar to the single muscle fiber action potentials recorded with the multielectrodes (Chapters 2 and 4)

In composite potentials of voltage 100–500 μV individual spike com ponents could usually be discerned often however only as humps or other irregularities in the waveform The jitter phenomenon was *usually* seen but the slow time course of the potentials could make the triggering of the sweeps unstable The whole oscilloscopic picture was then blurred due to technical reasons and it was then difficult to detect the jitter For this reason the jitter phenomenon was only rarely seen in action potentials of voltage less than 100 μV In other words The jitter was seen in all cases where the recording system permitted a detection of a variability in the waveform of an order of magnitude of 10 μsec (cf page 69)

It was far more difficult to keep the electrode in constant position in relation to the fiber when using concentric needle electrodes than when using electrodes at the side of a needle and movement artefacts were then more liable to obscure the jitter phenomenon

In 83 recordings the needle position was adjusted for optimum voltage of the recorded spike potential In all these recordings the optimum position proved to be that in which the electrode in the bevel surface of the tip faced the fibers The voltage distribution of these spikes is given in Fig 39 *B*

In another series of experiments the shaft of the needle was turned 90° as shown in Fig 39 *A* and kept so during the search for potentials The adjustment for optimum voltage was then made only by to-and fro adjust ments of the needle Fig 39 *A* shows the voltage distribution obtained for 70 spikes

The rise time for the spikes was of the order of 200 μsec and the spike duration (measured according to Fig 7) of the order of 500–1000 μsec, both parameters approximately twice as large as with a 30 μ electrode No statistical analysis of the influence of the needle position on the time course of the potentials has been made in this investigation

There seemed to be no substantial difference between the recordings in which the shaft of the needle was used as reference electrode and those in which separate subcutaneous reference was used

Discussion

As the jitter phenomenon is as clearly visible when recording with con centric needle electrodes as with the multielectrodes it may be stated that action potentials recorded with the former and free of jitter are single muscle fiber action potentials provided that in each individual case the recording system permits the detection of a variability in the waveform of the order of 10 μsec

These jitter free spike potentials seem to be the same sort of potentials as described by Buchthal *et al* (1957 *a*) as diphasic components of short duration and high amplitude and termed by them *the spike of the motor unit potential*

The lower voltage and slower time course of the single muscle fiber action potentials recorded with the concentric needle electrodes must be explained as an effect of the greater electrode area (cf page 74) but it is possible that the part of the shaft of the needle that surrounds the bevelled surface of the electrode is partly responsible It might be more difficult to come close to an active fiber with the electrode in the bevel surface than with the electrode at the side of the needle

The larger electrode area in the concentric needle electrodes also explains why single muscle fiber action potentials were more rarely encountered than when using the multielectrode With increase of the electrode area the chance

of there being more than one fiber belonging to a particular motor unit close to the electrode will increase

The increased voltage of the spike potentials when the bevel surface of the needle *faces* the fibers in comparison to when the needle is rotated 90 from this position is to be expected but it does not seem to have been taken into account in previous investigations

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The larger electrode area in the concentric needle electrodes also explains why single muscle fiber action potentials were more rarely encountered than when using the multielectrode With increase of the electrode area the chance

SUMMARY

The present investigation is a study of single muscle fiber action potentials in the normal human electromyogram. The possibility of recording such potentials has been questioned or denied. Action potentials of seemingly simple shape have often been recorded, but there has been no way to decide whether they were derived from single fibers or from aggregates of fibers. However, with the knowledge gained from the present investigation it is now possible to identify and study those potentials that are derived from single muscle fibers.

In *Chapter 1* the methods are described. Most experiments were made with a needle multielectrode with fourteen 30 μ platinum electrodes mounted flush with the side of the needle. The small electrodes permitted recording with but little distortion; the electrode arrangement made possible recording from several sites simultaneously. The action potentials were displayed on the oscilloscope with the sweep triggered by the action potential.

To define the characteristics of a single muscle fiber recording made with the multielectrode, a method was developed (*Chapter 2*) for chemical activation of single muscle fibers *in situ* by micro-injection of sodium citrate into the muscle. There are strong reasons to believe that muscle fibers chemically activated in this way are independently discharging. The most important property of these single fiber recordings was that all consecutive discharges were of identical shape.

In the voluntarily activated muscle the fibers are not independently discharging. The question then arises whether it is possible for two or more adjacent fibers to generate composite potentials that simulate those from a single fiber. This question is dealt with in *Chapter 3*, where a study has been made of *obviously composite* potentials in order to gain experience in what is to be expected from composite potentials where the composite character is not detected in the shape. Some quantitative aspects of this

problem were also treated by synthetic composite potentials obtained by adding simple potentials of known shape voltage and mutual time interval The most important finding was that composite potentials were never of equal shape at consecutive discharges There were small variations in the waveform the *jitter phenomenon* The cause of the jitter was found to be a variability of the order of 10–30 μsec in the mutual time interval between the spike components of the composite potentials The individual spike components were however of identical shape at consecutive discharges The jitter phenomenon was interpreted as being caused by a variability in the end plate delay of the individual muscle fibers

It was found that spike potentials that were free of jitter behaved like the chemically activated ones in all other respects as well and that such potentials could not have been generated by two or more adjacent fibers (all making appreciable contributions to the potential form) An interference from a remote spike exceeding 10 per cent of the amplitude of the main spike would also be detected by the jitter phenomenon Thus *spike potentials that are free from jitter are single muscle fiber action potentials provided the time resolution in the recording system is such as to permit the detection of a variability in the waveform of the order of 10 μsec*

In *Chapter 4* some parameters of the single muscle fiber action potentials are reported The action potentials had a clean smooth biphasic spike component often followed by terminal phases of low amplitude and long duration The median value for the voltage was 5 6 mV and the maximum value obtained was 25 2 mV The median value for the spike duration was 470 μsec These values are not representative for the total fiber population since the method favours the recording from larger fibers

In *Chapter 5* it is shown that single muscle fiber action potentials can also be recorded with concentric needle electrodes and identified by the absence of jitter

Buchthal Guld & Rosenfalck have concluded that the clean smooth spikes obtained during voluntary contraction are derived as a rule from small groups of perfectly synchronized tightly packed fibers subunits In *Chapter 6* results are presented which are not in accordance with the subunit concept Recordings consistent with a piercing of the subunit by the multi electrode were never obtained Non-simultaneous action potentials from closely adjacent fibers belonging to the same motor unit were however found

The present findings indicate intermingling of fibers belonging to different motor units

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ANALYSIS OF IONIC TRACER MOVEMENTS DURING SINGLE HEART CYCLES

BY

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It is the purpose of the present work to describe methods for and to present some results of the relation between ionic fluxes and electro mechanical activity of heart muscle (sinus venosus and atrium of the toad)

The *introduction* consists of a review of the background to these studies together with a general discussion of the problem involved

In the *methodological section* the preparation of the tissue sample used i e sinus venosus and in some cases atrium from toads is described This is followed by a description of the perfusion system and the recording methods used Under the titles of *influx experiments* and *efflux experiments* are given experimental results together with a discussion of methodological problems while the biological discussion is treated under the heading *general discussion*

CONTENTS

INFLUX EXPERIMENTS

EFFLUX EXPERIMENTS

GENERAL DISCUSSION

Guide for some abbreviations and special terms

AC component see pp 54–55
base line see p 54
CAT see p 53
cat see p 53
cpm counts per minute
cps counts per second
cps see p 81
Δcps_r, see p 81
Δcps_s see p 81
D diffusion coefficient
DC component see p 54
decimal pip method see p 39
direct monitoring method see DMM
DMM perfusion technique see p 45
DMM radioactive analysis see p 38
ESA equivalent specific activity see p 41
intercept interpolation method see p 57
kcpm 1 kcpm = 10^3 cpm
kcps 1 kcps = 10^3 cps
M molar (concentration)
M molar mass
$\overline{M}_n$ average molar mass (number average)
$\overline{M}_w$ average molar mass (weight average)
mean cycle see p 54
mean trend see p 54
monitoring pulse see p 39
N number of observations by DMM see p
n number of observations (for all experiments except DMM) (see p 22) number of recorded impulses for radioactive analyses (see pp 37 41)
O_R external surface film during silicone oil perfusion see pp 66–67
O_{RD} see pp 66–67
original value method see p 57
R Ringer solution (phase)
R long Ringer perfusion period
r short Ringer perfusion period
S silicone oil (phase)
S long silicone oil perfusion period
S standard error of the estimate
s short silicone oil perfusion period
sampling method see SM
SM see p 49
t_m length of radioactive measuring period see p 45
t_r length of Ringer perfusion period see p 45
trapped activity see pp 60–61
t_{eq} see p 74
t_s length of silicone oil perfusion period see p 45
$\bar{v}$ linear mean velocity see pp 29–30
v_{method} see p 37
V_o volume of O_R see p [illegible]
v_{stat} see p 37
x value of single observation
$\bar{x}$ arithmetic mean of observations x_1 x_2 ... x_n

INTRODUCTION

I The Purpose of the Present Investigation

BERNSTEIN (1902 1912) made the first attempt to explain the asymmetry in the distribution of the inorganic ions between the inside and outside of living cells As experimental methods have developed new and more preci e data for an ever increasing number of different tissues and cell types have been obtained making it possible to extend the theoretical treatment Particularly important has been the devel opment of electrophysiological recording methods (SCHUTZ 1936 HODGKIN and HUXLEY 1939 GRAHAM and GERARD 1946) which has made it possible to amass data for both resting and action poten tials

The structure in the cell membrane and the driving forces which work within it are the factors with which most theories attempt to explain *resting potential* in the cell membrane (for references see below) LING (1962) has presented however a hypothesis which is es entially based on the *properties of the cytoplasm* itself BOYLE and CONWAY (1941) have like BERNSTEIN presented a hypothesis concerning the properties of the cell membrane their postulates were based essen tially on studies of striated muscle Important studies of the electrical properties of the cell membrane were initiated by COLE and CURTIS (1939) who studied the membrane impedance during a single action potential GOLDMAN (1943) appears to be the first who treated the rectification properties of membranes theoretically Passive and active transport has been treated by USSING (1949*a* *b* 1951) The constant field assumption presented by HODGKIN and KATZ (1949) goes back to GOLDMAN s (1943) constant field theory and is an antecedent to the now well known HODGKIN–HUXLEY theory (1952*a–d*) TEORELL (1951 1953) treated this problem also in the case for a charged mem brane

According to the HODGKIN–HUXLEY *theory* (1952*a–d*) the ionic distribution *at rest* i maintained by an active outward transport of sodium coupled with a very low sodium permeability This theory is

based mainly on data obtained from the squid axon and explains resting potential as depending on the concentration differences be tween intracellular and extracellular ions mainly potassium ions which exist in the resting state

TEORELL (1960) put forward a hypothesis in which he postulated that the electro chemical and electro osmotic relationships led to a bulk flow which in its turn resulted in the separation of ions within the charged pores of the membrane According to TEORELL s view water transport between the cellular and extracellular spaces may play an important role in the genesis of the electrical events (cf TEO RELL 1962)

In order to explain the repolarization of the cell membrane which takes place during an *action* cycle in an excitable cell permeability changes within the cell membrane (causing a shift from K to Na do minance) have been postulated (HODGKIN and HUXLEY 1952*a–d*)

It is however not only events in nervous and striated muscular tissue which have attracted great interest During the last ten years much data has been obtained concerning relationships during excita tion in both the muscles and conducting system of the heart Since WOODBURY WOODBURY and HECHT (1950) first recorded intracellu larly membrane potentials of a single heart fibre much data concern ing the relationships of the heart muscle cell have been presented Further information may be obtained in recent review articles (BROOKS HOFFMAN and ORIAS 1955 WEIDMANN 1956 SCHUTZ 1958 CARME LIET 1961 TRAUTWEIN 1963)

From an analysis of published electrophysiological data NOBLE (1960 1962) developed a theory analogous to that of HODGKIN–HUXLEY which takes regard of the special electrophysiological con ditions in heart tissue A theoretical discussion of the Na and K hypo *thesis has been presented also by BRADY and WOODBURY (1960)*

Generally it may be said that most workers are agreed that the findings from a particular excitable tissue are applicable to most other types of excitable tissue

We shall now discuss the observations which support the view that there is a connection between the electrical activity and ion fluxes in excitable tissue

KEYNES (1951) and KEYNES and LEWIS (1951) studied ion fluxes (Na^+ K^+) in nerve tissue and found that the rate of accumulation of the tracer in an axon was higher during stimulation than at rest

the outflow of the tracer was also more rapid when the nerve was being stimulated Similar results were obtained by TASAKI TEORELL and SPYROPOULOS (1961) who studied Na^{24} and K^{42} outflow from squid axon the rate of outflow of the tracer depending on the stimulation frequency In studies of cardiac muscle SCHREIBER (1956) found that the K^+ outflow was related to the cardiac work done similar findings were reported by GRUPP (1963) Reference should also be made to SEKUL and HOLLAND (1959) who studied Na^{24} K^{42}, Ca^{45} and Cl^{36} fluxes in isolated rabbit atria and obtained an increased influx of Na^{24} Ca^{45} and Cl^{36} and an increased K^{42} and Cl^{36} efflux with stimulation (no effect of stimulation on Na^{24} and Ca^{45} effluxes) HARRIS (1958) observed that stimulation also increased Cl^- output from frog muscle The potassium permeability in Purkinje fibres was studied by *CARMELIET (1961) HAAS CLITSCH and TRAUTWEIN (1963) found* that the rate of efflux of Na^{24} was greater from a beating than from a non beating heart

It is further known that acetylcholine and vagus stimulation influence ion transport in the heart BURGEN and TERROUX (1953) showed that acetylcholine and similar substances acted on the heart by increasing the permeability for potassium ions This was confirmed among others by SEKUL and HOLLAND (1959) HUTTER (1961) and DANIELSON (1962 1964) FATT and KATZ (1951) interpreted changes in the motor end plate potential in frog muscle by assuming that acetylcholine produced a non selective increase of ionic permeability KLEIN and HOLLAND (1958) claimed that acetylcholine increases the trans membrane flux of Na^+ and K^+ (studies of Na^{24} and K^{42} on isolated rabbit atria) SEKUL and HOLLAND (1959) obtained no effect of acetylcholine on the fluxes of Ca^{45} and Cl^{36} in isolated rabbit atria DANIELSON (1964) found an increased potassium transport in the toad heart as a result of vagal stimulation but no effect on Na^+ or Br^- ions was observed These latter observations concerning bromide are in agreement with HUTTER (1961) WADELL (1961) studied potassium movements in auricular muscle under the influence of adrenaline which he claimed increased both influx and efflux of potassium HAAS and TRAUTWEIN (1963) reported an increase of sodium efflux induced by adrenaline in the heart of frog

Several workers have studied influx and/or efflux rates for sodium and potassium HARRIS (1957) studied K^+ movements in frog muscle as did HODGKIN and HOROWICZ (1959) BURROWS and LAMB (1962)

studied Na^+ and K^+ fluxes in tissue cultures of heart muscle from chicken embryo. Potassium movements in heart muscle have also been studied by PAYNER and WEATHERALL (1959) and HUMPHREY and JOHNSON (1960).

Data about the calculated mean flux (net value of simultaneous inward and outward fluxes) for the ions studied during the whole cycle of activity given as the number of micromoles transported per unit of time or per activity cycle and weight of tissue can be found in many of the works cited above (for a review reference may be made for example to CALMFLIET 1960 p. 26 ff.). *Calculations of the quantitative fluxes* from tracer experiments may be misleading if there is some uncertainty about the question of maintenance of a uniform specific activity during the experiment.

The experiments discussed above cannot give information about the *direct* relationships between the ion flux and the different phases of a *single* action cycle. It seems clear that is particularly desirable to obtain detailed information about this *direct relationship between ion flux and the single action cycle*. Experiments with this aim have been carried out, for example on squid axon by SPYROPOULOS and TASAKI (1961). It appears probable that such information can also be obtained on other excitable tissues. Only some isolated attempts to determine the ion flux during a single action cycle in heart muscle have been published. The experiments of WILDE and O'BRIEN (1953) are well known; the studies were made on isolated turtle heart and an increased K^+ efflux was found during the later part of the T wave of the electrocardiogram. DANIELSON, ÖBRINK and SJOSTRAND (1961, 1962) studied Na^+ and K^+ efflux from an isolated auricle and/or sinus venosus of the frog. Na^{24} and K^{42} effluxes showed cyclical variations in relation to the heart cycle. Na^{24} efflux had a maximum at the end of the electrical activity while the K^+ efflux appeared to be increased during the whole of the electrical activity. LOIBER *et al.* (1962) studied K^+ efflux from a piece of frog ventricle and found an increased efflux during and of approximately the same duration as the action potential.

The present work attempts a more detailed and more *direct* qualitative analysis of ionic flux in an excitable tissue (heart muscle) during the single action cycle (electro mechanical). The possibilities for such an analysis depend to a great extent on matters of a purely technical nature. The question of the selection of the experimental

preparation and the general principles of attacking the problem must first be considered

The ideal preparation should have a long action potential which for example, by cooling can be further prolonged It should further have a good surface volume relationship in order to obtain good exchange conditions it should also be of a suitable size and sufficiently large for measurements to be made (this latter factor involves a compromise with the former) *The sinus venosus in the toad* seemed to be a rather suitable experimental object though it has a large extra cellular space and like the other parts of the heart mechanical activity both these properties complicate the study of membrane processes From a general methodological point of view the sinus venosus and/or auricle of the toad were considered to be suitable both these cardiac regions are easily prepared and have a good viability

The only possibility of studying *radioactive influx* in a preparation is to follow the changes in the radioactivity in the preparation itself Studies of the influx of ions have been published (HARRIS 1953 JOHNSON 1957 SHANES 1958 CARMELIET 1961 HAAS GLITSCH and TRAUTWEIN 1962 1963 TASAKI 1963) The methods reported however cannot be used if it is desired to analyse the radioactivity of the preparation under time intervals so short that several observations can be made during a single cycle of activity (for example a single heart cycle)

In *studying the radioactive influx* the preparation must on the one hand have the possibility of taking up radioactive tracer and on the other hand the measurement of its radioactive content must take place when it is not present in a radioactive bathing solution The problem is such that the preparation should be in contact with a radioactive bathing solution of high specific activity (the background activity $\gg$ the activity of the preparation) and during very short time intervals it should be analysed for its radioactive content when the background activity is very low (the background activity $\lesssim$ the radioactivity of the preparation) The principles for such a discontinuous analysis together with preliminary results have been discussed by SJOSTRAND (1962 1963) further the problem has also been discussed by DANIELSON and SJOSTRAND (1963) If the preparation is mounted in a perfusion chamber (see Fig 19 p 38) which in its turn is placed in a NaI(Tl) crystal the gamma activity can be analysed continuously By alternate perfusion with radioactive Ringer s

solution (high specific activity) and a low viscosity silicone oil (hydrophobic and not radioactive) the preparation comes into contact with the isotope during the Ringer perfusion period while during the silicone oil perfusion period it is surrounded by silicone oil in the perfusion chamber whereby the background activity is reduced to a very low level During the silicone perfusion period the radioactivity recorded is mainly that present in the preparation By a continuous recording of the electrophysiological events in the preparation (in these experiments the ECG) the discontinuous radioactivity measurements can thus be analysed in terms of the electrical events

Studies of the radioactive efflux are technically much simpler The pioneer work was made by WILDE and O'BRIEN (1953) The efflux studies discussed above have all been made in technically similar ways and are all in principle different modifications of the wash out technique (DANIELSON ÖBRINK and SJOSTRAND 1961 1962 LORBER *et al* 1962) This method was also used by SPYROPOULOS and TASAKI (1961) on squid axon

II The Sinus Venosus and the Atrium of the Heart of the Toad

Sinus venosus or atrium from the toad (*Bufo bufo*) was used Concerning the anatomical details reference should be made to ECKER-WIEDERSHEIM (1904 p 269) SKRAMLIK (1921) and UCHIYAMA (1959) Fig 1 shows a semi schematic diagram of the toad heart

The main object for the present studies has been the sinus venosus but when it was desired in a simpler way to study the normal physiological relationships the atrium has also been used (the electrical and mechanical properties studies of the equilibrial volumes and the selection of suitable test molecules for such studies) In addition use of the atrium and sinus venosus enables more certain comparisons to be made with the results reported in the literature

a) Microscopic anatomy

A short review of the microscopic anatomy has been given by KISCH (1961) who maintains that the *sinus venosus* has a layer of striated muscle which has similar histological properties as the muscle in other parts of the toad heart The endothelial cells have a large number of mitochondria (similar to the mammalian heart) Between the epithelial cells and the muscle cells are a large number of longitudinal and transverse collagen fibres Capillaries are never found between the muscle cells In a later work (KISCH 1962) it was shown that the endothelial layer in certain places has a thickness of only 0 1 μ or less and in certain places the sarcolemma of the nearest muscle fibres was separated from the endothelial covering cells by a subendothelial stratum The protofilament in a muscle cell has a diameter of about 180 Å The *atrium* has to a great extent a similar construction the muscle cell layer is however considerably thicker LINDNER (1957) reported the thickness of the sarcolemma to be 150–200 Å for ventricular muscle of *Rana temporaria* and claimed that the endothelial cells correspond to the vascular endothelium The extracellular

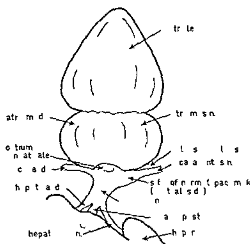


FIG 1 Toad heart (*Bufo bufo*) semidiagrammatic To expose the sinus venosus the auricles and ventricle have been lifted cranially

space begins in the subendocardial region and communicates with tissue spaces between the muscle cells The heart muscle cells have a greatest thickness (in the region of the cell nucleus) of between 2 5 and 6 μ their diameter however can be as low as 0 45 μ elsewhere In cross section the muscle cells consists to about 2/3 of fibrillae and to about 1/3 of cytoplasm Morphologically the boundary between the intra cellular and extracellular spaces is difficult to define A value of less than 5 μ was given by HUTTER and TRAUTWEIN (1956) for the heart muscle cells in the frog and by IWAMOTO (1959) for sinus venosus fibres in the toad HAAS GLITSCH and TRAUTWEIN (1963) give a diameter of about 4 μ for atrial muscle cells in the frog

Briefly may also be mentioned the interesting observations of SIMPSON and OERTELIS (1962) that in the sarcolemma of the sheep heart the surface membrane complex seems to be indented with each Z band in the subsarcolemmal myofibrils There appeared to be two different tubular systems within the cells (sarcotubular systems) of which one had relatively thick walls which were identical with the sarcolemma while the other had thinner walls The thick walled tubules seemed to be invaginations of the sarcolemma which passed right across the cells In serial sections it was possible to show that this tubular system was in continuity with the extracellular space The transverse tubules and their communication with the extracellular space imply not only advantages for the propagation of the im



FIG. 2. Histological section of an inverted sinus venosus (left) and atrium (*Bufo bufo*). The tissue was fixed in Bouin's solution, dehydrated and embedded in paraffin. 10 μ thick section stained with hematoxylin and eosin. × 200

pulse, but also that the total area of the sarcolemma must be considerably greater than would appear from the external area of the cell itself. This might mean that large ion fluxes could occur in a very short time. In what way the extracellular fluid perfuses the tubular system is however unknown. SPIRO (1962) has also observed invaginations in the sarcolemma.

In certain muscle tissues nearly all myofibrils are in contact with the external sarcolemma, for example in cells which have a very small diameter, i.e. toad myocardium (FAWCETT and SELBY, 1958; GRIMLEY and EDWARDS, 1960). In these cases it would appear that a tubular system of the type present in the mammalian heart was not required.

Fig. 2 shows the general morphology of an inverted sinus venosus and atrium from a toad (*Bufo bufo*).

b) *Preparation of the toad heart*

After decapitation of the toad the heart was dissected free and the sinus venosus and/or atrium was separated from the surrounding connective tissue. Both the anterior venae cavae were ligated within a few mm of the entrance into the sinus venosus. In a preparation of the latter it was separated from the atrium by cutting along the circular sulcus (Fig. 1). A plastic catheter was introduced through the sino-atrial

orifice and passed out through the posterior vena cava Both hepatic veins were ligated The sinus venosus could then be cut out from the toad and was then transferred from the plastic catheter to the ECG lead catheter as shown in Fig 22 (p 46) which is fixed to the part of the U tube which served for both influx and efflux experiments (see chapter V) The sinus venosus was thereafter turned inside out (the endothelial side = the outside) and secured to the catheter at both ends When being fixed to the ECG catheter in the perfusion chamber the sinus venosus was stretched a little so that the amplitude of the contrac tions was reduced considerably The endothelial side of the sinus venosus could with this technique be washed without variations in tension occurring in the wall of the preparation (a rise in tension in the wall of the sinus venosus increases the spontaneous activity cf PATHAK 1958 MAINWOOD 1962) When the atrium was used the preparation was made in a similar way i e it was dissected free from the bulbus arteriosus A cut was then made along the atrio ventricular groove after which the catheter could be pushed in through the right atrium and out through the sinus venosus The preparation was then cut along the circular sulcus as before [1] The atrium was transferred in a similar manner to the ECG catheter in the perfusion chamber turned outside in (left atrium became situated in the inverted right atrium) and secured to ECG catheter In this case also the preparation was stretched in order to reduce movements during contraction

The sinus venosus or atrium prepared in this way beat spontane ously In normal oxygenated frog Ringer s solution (solution I see below) spontaneous beating continued for as long as several days In all experiments the tissue was allowed to rest for about 30 minutes after the preparative manipulations in order to allow the potassium flux from the damaged cells to cease (BAMMER and POT SCHUH 1962a b ROTSCHUH 1962) and also that the healing over could have time to take place (DÉLÈZE and WEIDMANN 1963)

The size of the tissue preparation is given as a rule as follows in terms of the wet weight The length mounted on the ECG catheter was as a rule 9–10 mm The diameter varied from 1 5 mm (sinus venosus) to 2 5 mm (certain atrial preparations)

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[1] An atrial preparation will only beat spontaneously with an intact cir cular muscle bundle at the sino atrial junction this is part of the sino atrial conduction system of the amphibian heart (UCHIYAMA AKUZAWA and IWA MOTO 1961)

c) *Normal physiology*

Nutrition

The toad heart has no coronary system and the heart muscle normally obtains its nutriment by a direct transport of soluble substances from the plasma in the heart lumen through the sponge like structure of the wall. Perfusion of the endothelial side of the sinus venosus or the atrium simulates therefore the normal way of nutrition for the cells of the preparation. The geometric conditions in particular for the sinus venosus seem to fulfil the demand as stated by HILL (1928) for oxygen supply to an actively working muscle preparation.[1] PAGE and SOLOMON (1960) give for cat heart papillary muscle the value of $r_0 = 0.6$ mm ($+37°$C) (see footnote). In the avascular sinus venosus the relations must therefore be even better since the total wall thickness is as a rule of the order of 100 μ (see Fig. 2, cf. also HUTTER 1961). KEYNES (1954) points out that with K studies the preparation should not have too large dimensions.

The site of the pace maker

Already in 1897 ENGELMANN studied where the heart contraction began. IWAMOTO (1959) reviewed the studies on the localization of the primary pace maker in the sinus venosus and reported studies which suggested that it was localized to the ventral part of the opening of the left anterior vena cava into the sinus venosus (see Fig. 1). HUTTER and TRAUTWEIN (1956) claim that in the amphibian heart (toad) the primary pace maker is normally situated in the left side of the ventral part of the sinus venosus.

The resting and action potentials, ECG[2] and mechanical activity

The resting and action potentials of the preparation at the perfusion temperature used with influx and efflux experiments were

[1] The equation derived by HILL gives the maximal radius of r_0 for a cylindrical muscle which receives an adequate oxygen supply through the diffusion through the medium as

$$r = \sqrt{4ky_0/a}$$

where k is a constant for the diffusion of oxygen through the muscle (cm^2 min^{-1} atm^{-1}), $k = 1.4 \times 10^{-5}$ at 0 °C and increases about 1 per cent per degree C (KROGH 1919), y_0 = the concentration of oxygen in the medium (the atmosphere), a = the rate of consumption of oxygen in ml/cm^3 tissue per min.

[2] ECG as used here means the electrical activity recorded with a surface electrode (surface electrogram).

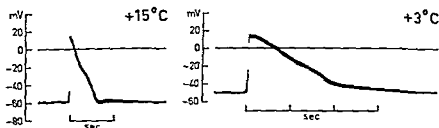


FIG 3a Membrane potential (intracellular micro electrode) from a cell in an inverted spontaneously beating sinus venosus (non pace maker region) of *Rana pipens* Temperatures +15 C and +3 C

studied Figs 3a and 3b show an intracellular recording of the action potential in an inverted spontaneously beating sinus veno us from *Rana pipens* (two different cells in the same preparation) The record ings in Figs 3a and 3b are in essential agreement with those reported in the literature (HUTTER and TRAUTWEIN 1956 IWAMOTO 1959 UCHIYAMA AKUZAWA and IWAMOTO 1961) For recording the action potential in the tissue preparation used for influx and efflux studies a simple type of suction electrode was used (see chapter IV A c 2) Fig 4 shows such a recording of a spontaneously beating sinus veno sus The temperature dependence of the diastolic depolarization can be clearly seen in the figure (cf TRAUTWEIN GOTTSTEIN and FEDER SCHMIDT 1953 CORABOEUF and WEIDMANN 1954) In order to obtain a longer action potential the perfusion was cooled in the ion flux experiments which will be described Cooling[1] leads to a lower resting potential a longer plateau in the action potential which how ever is not essentially altered in other details (WOODBURY HECHT and CHRISTOPHERSON 1951 TRAUTWEIN GOTTSTEIN and FEDER SCHMIDT 1953 HEINTZEN 1954 HECHT 1956 WEIDMANN 1956 HEINTZEN KRAFT and WIEGMAN 1956)

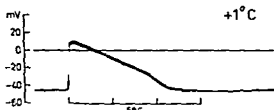


FIG 3b Membrane potential (intracellular micro-electrode) of another cell in the same preparation as Fig 3a Temperature +1 C

[1] The efflux of K^{42} from non beating atria is less at a lower temperature of the soaking solution (HAAS and GLITSCH 1962)

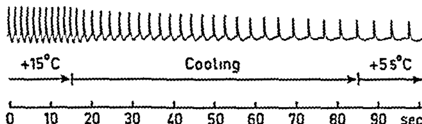


Fig 4 Action potential (suction electrode) of an isolated inverted spontaneously beating sinus venosus (pace maker or near pace maker region) during cooling from +15 C to +5 5 C

During an influx or efflux experiment only the ECG was recorded but from that the length of the action potential could be deduced This can be seen in Fig 5 which shows an ECG and action potential (suction electrode technique) in a spontaneously beating sinus venosus and in Figs 6 and 8 on a spontaneously beating atrium The relationship between action potential and ECG has been treated by HECHT (1956) and SANO TSUCHIHASHI and SHIMAMOTO (1960) among others

The mechanical activity of the preparation was also of interest Fig 6 shows the ECG action potential (suction electrode) and the mechanogram (isometric) of a spontaneously beating atrium Fig 7 shows similar recordings from a spontaneously beating sinus venosus The mechanical activity of the ventricular muscle of *Rana temporaria* has been described by HEINTZEN (1954) and HEINTZEN KRAFT and WIEGMAN (1956)

Table I (p 22) summarizes the data regarding the duration of the action potential (measured by the suction electrode) the time between

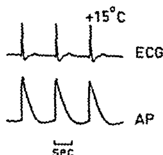


Fig 5 Simultaneous recording of ECG and action potential (suction electrode) from an isolated inverted spontaneously beating sinus venosus

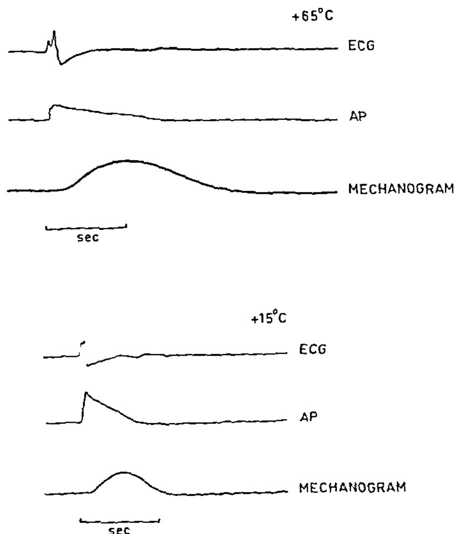


Fig 6 The ECG action potential (suction electrode) and mechanogram (isometric) of an inverted spontaneously beating atrium at +6 5 C and +15°C

depolarization (assessed from the ECG) and the beginning of the mechanical activity and also the time between depolarization and the maximum contraction of the inverted atria In the case of the inverted sinus venosus of the toad the time interval between depolarization (ECG) and the beginning and the maximal values of the mechanical activity are given The durations of the action potentials in the atrium are somewhat shorter than those given in the works cited above where the investigations were performed on the ventricle musculature

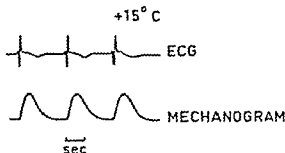


FIG " The ECG and mechanogram (isometric) in an inverted spontaneously beating sinus venosus

Preliminary investigations on the ventricle muscle with the methods used here gave however somewhat higher values for the duration of the action potential It seems therefore that the action potential in the preparation used is in fact somewhat shorter than in ventricular muscle Shortening of the duration of the action potential with oxygen deficiency is known (TRAUTWEIN GOTTSTEIN and DUDEL 1954) an explanation which can be rejected in this case

Has the isolated heart preparation a synchronous electrical activity?

Since the aim was to study ion fluxes in relation to electrical and mechanical activity it was of great importance that the electrical activity of the preparation should be synchronous in all its parts Since as mentioned above the sinus venosus has a pace maker localized at the site of the entrance of the left anterior vena cava it seems probable that impulse propagation (propagation of depolarization) occurred through the preparation Two questions were thus of interest

1 The conduction velocity of the preparation (the conduction velocity of linear propagation through the preparation) and

2 The relationship between the ECC and the action potential in different parts of a spontaneously beating preparation (propagation of depolarization from a pace maker whose location is not known in any particular case)

The conduction velocity of the preparation was determined by a method which is described in chapter IV D b and the values for three isolated atria are given in Table 2 HEINTZEN (1954) reported at +5 5 C in ventricle muscle *in the frog* a conduction velocity of 5 cm per sec HEINTZEN s value agrees with that given by BENTHE

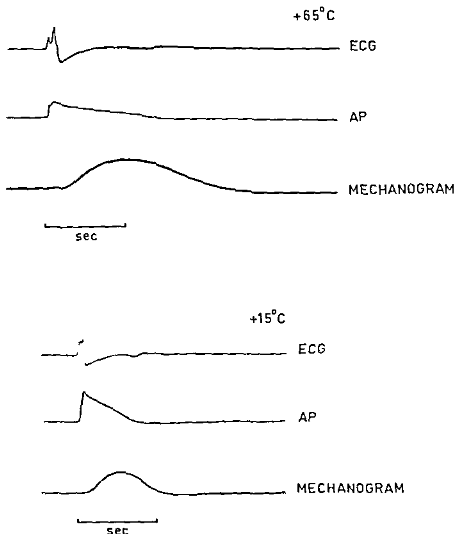


Fig. 6 The ECG action potential (suction electrode) and mechanogram (isometric) of an inverted spontaneously beating atrium at +6.5 C and +15 C

depolarization (assessed from the ECG) and the beginning of the mechanical activity and also the time between depolarization and the maximum contraction of the inverted atria In the case of the inverted sinus venosus of the toad the time interval between depolarization (ECG) and the beginning and the maximal values of the mechanical activity are given The durations of the action potentials in the atrium are somewhat shorter than those given in the works cited above where the investigations were performed on the ventricle musculature

TABLE 2 *Conduction velocity measured at + 5 5 C (isolated atrium)*

Exper No	Minimal conduction velocity (cm/sec)	No of deter minations	Range
630327	7 5	11	6 0–10 0
630328	6 0	26	4 6–10 0
630410	6 8	37	4 3–10 3
Mean	6 8		

This table gives a summary of results from isolated spontaneously beating atria which were stimulated electrically after approximately each 5th spontaneous beat 1–2 sec before the next expected would normally occur Propagation of depolarization recorded with suction electrodes (chapter IV D b) Each experiment represents measurements on one preparation

(1956) The only value of the conduction velocity of the atrium is that of ENGELMANN (1895) i e 9 cm per sec at room temperature

The relationship between ECG and action potential in different parts of an inverted spontaneously beating atrium is shown in Fig 8 The

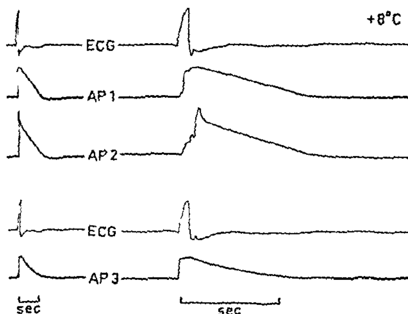


FIG 8 The ECG and action potential (*AP* suction electrode) of an inverted spontaneously beating atrium at two different paper speeds (for method see Fig 15 p 34) *AP* 1 and *AP* 2 were recorded at the same time as the ECG above The lower ECG was taken later at the same temperature when *AP* 3 was recorded

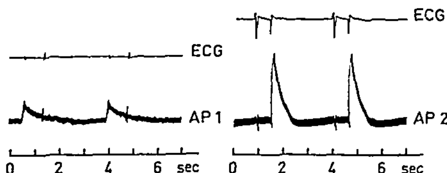


FIG 9 The ECG and action potential (suction electrode cf Fig 15 p 34) from an inverted spontaneously beating atrium The left ECG and AP 1 were recorded during Ringer perfusion and the records on the right during silicone oil perfusion

figure shows simultaneous recordings of the ECG and action potentials from the upper (AP 1) and lower parts (AP 2) of the preparation in the lower curves the ECG was also taken from a combined action potential lead (AP 3) with the method described in chapter IV D b Fig 15[1] It can be seen that depolarization occurred practically simultaneously in the whole preparation That the method used in the experiment of Fig 8 was sufficiently sensitive to resolve two different electrical activities in the same preparation is evident from Fig 9 which shows suction electrode recordings from the upper (AP 1) and the lower (AP 2) parts of another preparation with simultaneous ECG recording This preparation had two electrical activities one resulting from the primary pace maker and which clearly triggered the low parts of the preparation AP 1 was recorded during Ringer perfusion and AP 2 during silicone oil perfusion[2]

There seem to be good grounds for accepting that the isolated spontaneously beating atrium or sinus venosus (preliminary studies showed that similar relationships applied to sinus venosus) exhibits very nearly synchronous electrical activity over the whole preparation

[1] The experiment of Fig 8 gave a conduction velocity of [illegible] cm per sec at [illegible] C when the preparation was stimulated in the upper part according to the method which is described in chapter IV D b When the preparation was allowed to beat spontaneously the result shown in the figure was obtained

[2] With regard to Fig 9 attention should also be drawn to the differences in ECG registration obtained with the Ringer perfusion (preparation surrounded by a volume conductor) and that obtained with silicone oil perfusion (preparation surrounded by an insulator)

Further it is apparent (see Fig 9) from the ECG recording whether the preparation has one or more electrical activities

Summary The macroscopic and microscopic anatomy of the sinus venosus and atrium in the frog and toad are reviewed The electrical activity appears to be synchronous in all parts of the preparation even at a low temperature (about +5 C) The mechanical activity also appears to be well defined From the ECG of the preparation the length of the electrical activity can be deduced

III The Perfusion System

As was briefly mentioned in chapter I in the influx experiments the preparation was alternately perfused with a radioactive Ringer solution and a low viscosity silicone oil If the latter has a linear flow rate sufficiently great to give a strongly turbulent flow it would be expected to wash away the greater part of the Ringer solution which remained around the preparation

In both influx and efflux studies the preparation was mounted as described in chapter II b on an ECG catheter (diameter 1 2 mm) which was fixed in a polyethylene U tube as shown in Fig 22 (p 46) With an influx experiment the perfusion system was arranged in such a way that the preparation was situated in a long U loop of polyethylene tubing which constituted a perfusion chamber (Fig 10)[1] In an efflux experiment the preparation was mounted as shown on the right side of the figure (Fig 22) and was perfused in the opposite direction to that with an influx study

In both influx and efflux studies the perfusion system shown schematically in Fig 11 was used R_1 R_2 and S are storage vessels for inactive Ringer solution active Ringer solution and silicone oil respectively From each storage vessel (polyethylene) a polyethylene tube passes through a counter current cooler to the two way tap which can be seen in the lower part of the figure In both influx and efflux experiments perfusion was performed with a high (constant) linear mean velocity In addition in the influx studies an alternating perfusion with Ringer solution and silicone oil could be made On these grounds the type of perfusion system shown in Fig 11 was used The perfusant was driven through the system by air pressure which was mediated through a tube system in connection to the storage vessels R_1 R_2 and S The air pressure used was maintained by a reducing valve at a level of about 1550 mm Hg (absolute pressure)

[1] The inner diameter of the perfusion chamber was 3 mm in the part where the preparation was placed (sinus venosus) the tubes leading to this part of the perfusion chamber had an inner diameter of 1 7 mm

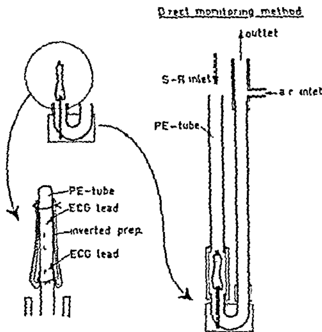


Fig 10 The perfusion chamber used for the direct monitoring method (DMM) For further detail cf legend of Fig 2 (p 46)

and held constant with a 735 mm high mercury column which in the event of an over pressure allowed the air to leak out of the system

The alternate Ringer silicone oil flow periods in the influx experiments were obtained by a rapidly working two way valve which is shown diagrammatically in Fig 12 The valve had inlets for both silicone oil and Ringer solution and as shown in Fig 11 the *inlet R* could be used for either inactive or radioactive Ringer solution The valve (made of perspex) had an easily movable piston which was driven by two serial coupled DC solenoids at each end with a combined effect of 0 5 kp (type TGB 4 Firma Magnet Schultz GmbH West Germany) The valve system could be washed through the *by pass outlet B* and test samples of Ringer solution taken during the silicone oil perfusion period The two way valve was operated electronically (see Fig 20 p 39) The shortest perfusion period obtainable with this valve was 50 msec

In an influx experiment the Ringer solution and silicone oil which had passed the preparation in the U tube were collected in a collecting

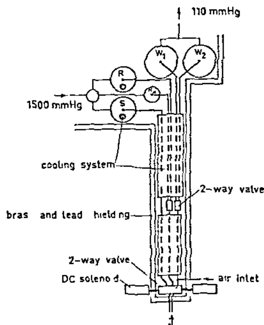


FIG 11 Schematic drawing of the perfusion system (supply and effluent for perfusing chamber) used in both influx and efflux experiments The pressures given are the absolute pressures in mm Hg used in influx experiments The storage vessels are S (silicone) R_i (inactive Ringer) and R_a (active Ringer) W_1 and W_2 are the two return vessels The latter were disconnected in an efflux experiment where the effluent from the chamber was led directly to sampling tubes in a fraction collector Further operating details are given in the text

vessel via a return tube In the efflux experiments the perfusate was collected in test tubes mounted in a fraction collector The return system used in the influx experiments is shown schematically in Fig 11 where two waste collecting vessels (polyethylene) W_1 and W_2 are drawn To obtain as far as possible similar perfusion and pressure conditions with both influx and efflux studies for the same perfusion pressure in the *Ri Ra S* system a sucker was applied in the return system in the influx experiment In order to minimize the radioactivity in the return tubes (to reduce the background activity during the influx experiment) they were in communication with the atmospheric pressure (air inlet in Fig 11) By this means and with the under pressure applied to the return system the increased resistance to flow in this part of the system can be compensated

To obtain a low background in an influx experiment it is necessary that practically all activity should be some distance away from the

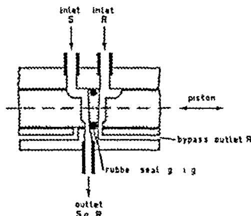


Fig 1. Diagram of the two way valve giving alternate Ringer silicone oil flows The piston movement required to switch over was 3 mm

scintillation crystal The supply vessels for the radioactive solution and the return vessels for both Ringer and silicone oil were therefore situated at an adequate distance from the crystal All supply and return lines were of minimal dimensions (polyethylene tube) For health reasons the radiation shielding must also be sufficient for large amounts of activity (about 50 mC Na^{24} with a high peak at 2 76 MeV) and also protect against the highest energy beta radiation (K^{42} 3 6 MeV) All radioactive containing vessels and lines were first sheathed in brass (3000 mg/cm²) which absorbs all beta energies under 4 0 MeV and round this lead bricks (55 000 mg/cm²) To lessen the danger of radioactive dissemination with a leakage or a possible explosion in the system all tubes were double walled

In order to obtain an idea of the physical relationships obtaining in the perfusion chamber during an influx study the linear mean velocity of the Ringer solution and silicone oil during perfusion was studied at different temperatures For an estimate of how the perfusion velocity varied depending on the size of the preparation experiments were made both with and without the preparation mounted in the perfusion chamber The velocities of the Ringer silicone and silicone Ringer phase boundaries (acceleration deceleration process) were also studied both in the presence and absence of the preparation (see chapter IV D a) From Fig 13 it can be seen that the linear mean velocity (in the incoming tube) with pure Ringer or silicone oil perfusion was about the same when the perfusion chamber was empty

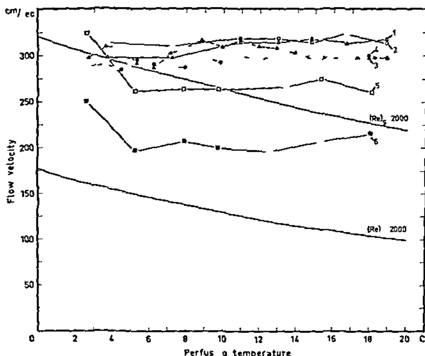


FIG 13 The mean linear velocities ($\bar{v}$) of Ringer solution and silicone oil in the supply lines to the perfusion chamber mounted as shown in Fig 22 (p 46) for the direct monitoring method The pressures were as shown in Fig 11 The mean flows were

without tissue preparation in the chamber	(1) Ringer solution $\bar{v} \approx 311$ cm/sec (2) Silicone oil $\bar{v} \approx 316$ cm/sec
with a sinus venosus (wet weight = 20 mg) in the chamber	(3) Ringer solution $\bar{v} \approx 303$ cm/sec (4) Silicone oil $\bar{v} \approx 294$ cm/sec

The velocity of the Ringer silicone phase boundary was determined as illustrated in Fig 21 (p 43) without a preparation (curve 5) and with a preparation (curve 6) The critical mean velocities for appearance of turbulent flow in an ideal tube system containing Ringer solution (Pe_R) or silicone oil (Re_S) are given there A mean flow velocity of $\bar{v} \approx 300$ cm/sec corresponded to a volume flow of about 7 ml/sec

as when a preparation was mounted in it At $\bar{v} \approx 300$ cm/sec the perfusion volume was of the order of 7 ml/sec The flow rate did not appear to vary significantly with the temperature The pressure conditions in the perfusion system were as shown in Fig 11 (p 28) Fig 13 shows that the Ringer silicone phase boundary velocity was greater with the perfusion chamber empty It may be concluded that viscosity changes of Ringer solution or silicone oil at different temperatures had no importance in the perfusion system

Fig 13 also shows the critical mean velocity for Ringer solution

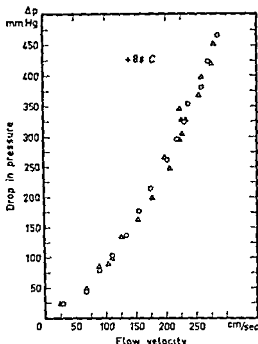


Fig 14 The pressure difference (Δp) between the part of the perfusion chamber where the tissue was mounted and the air inlet in Fig 11 (p 28) in relation to the mean linear flow velocity ( ) for Ringer solution (triangles) and silicone oil (circles)

and silicone oil at which turbulent flow appears in an ideal tube system (Reynold s number = 2000)[1] It can however be expected under all circumstances that there is a turbulent flow in the perfusion chamber with influx studies since all non ideal systems have a tendency to give turbulent flow (Reynold s number approaches unity)

Fig 14 shows the pressure drop (Δp) between that part of the perfusion chamber in which the preparation is mounted and the air inlet in Fig 11 (p 28) in relation to the linear mean flow velocity (v) for silicone oil or Ringer solution (the flow velocity is determined as in chapter IV D a 1) According to KRONIG (1954) the flow in the perfusion chamber at a perfusion velocity of about 300 cm/sec is in

[1] In calculating these lines the data for Ringer solution (approximately = water) were obtained from the Handbook of Chemistry and Physics ed 41 p 2181 and for silicone oil (3 cS at + 25 C) from the Midland Silicones Ltd Technical Data Sheet G 1 ed 6 June 1961

that part of the pressure difference–flow velocity diagram which corresponds to strongly turbulent flow ($\Delta p \propto \bar{v}^2$)

The temperature of the perfusion system was regulated with an accuracy of $\pm 0\ 1$°C and the maximal temperature difference between the inactive or active Ringer solution and the silicone oil during alternate perfusion was 0 2°C

The silicone oil used was a polydimethyl siloxane (Midland Silicones Ltd MS 200 2–3 cS at +25°C) Further information concerning this can be found in the Midland Silicones Ltd London England Technical Data Sheet G1 ed 6 June 1961 and only certain essential relevant properties will be discussed here Compared with organic fluids the silicone oil is very stable and has a density at +25°C of about 0 87 for 2 cS (M = 385 cf sucrose = 342) It has a very low surface tension a vapour pressure of 1 mm Hg at about +55°C and is strongly hydrophobic (non polar liquid) its solubility in water being less than 0 2 % Because of its very marked hydrophobic properties silicone oil does not dissolve ions (see DANIELSON and SJOSTRAND 1963) Its solubility for gases is good and in general the solubility is better than in water (for air the factor is about 10) The specific resistance is so high that it can be regarded as an insulator Among the chemical properties of silicone oil it may be mentioned that it has a neutral reaction and is very resistant to oxidation The toxicity of the oil used in these experiments is very low (ROWE SPENCER and BASS 1948) The toxicity for red blood cells has been studied by LINDOPPEN and ZADE OPPEN (1964) no hemolysis occurred when red cells were shaken together with silicone oil Its low toxicity is evident also by the fact that in all experiments that were carried out with Ringer silicone oil perfusion no toxic effect on the heart preparation was observed

IV Methods

A General recording methods

a) The ECG

The ECG lead was taken from the epicardial surface of the preparation (see Fig 15 p 34 the position of the ECG catheter in the perfusion chamber is shown in Fig 22 p 46) The ECG was then recorded via a Grass ECG preamplifier 5P4 (time constant 0 2 sec Figs 15 20) and a Grass Model 5 Polygraph Pen Recorder

b) The mechanogram

The preparation was mounted on the ECG catheter as shown in Figs 15 (p 34) and 22 (p 46 sampling method) That part of the perfusion chamber which led away the perfusant in efflux experiments was not connected the Ringer solution was able to run away over the surface of the preparation

For measurement of the mechanogram a thin nylon thread was fastened to one end of the preparation This thread passed to a thin metal strip on each side of which two strain gauges were fixed (the metal strip was held fast on a stand) These strain gauges functioned as the arms in a Wheatstone bridge which was supplied with a stable direct voltage The movements of the metal plate could thus be transduced to changes in potential which were registered with the Grass Polygraph recorder The response of the system was linear within the region used

c) The action potential

1 *Micro electrode technique*

This part of the work was carried out in Dr H Antoni s laboratory in the Institute of Physiology (Head Professor A Fleckenstein) University of Freiburg i Br West Germany The apparatus and method was as described by Antoni Engstfeldt and Fleckenstein (1960)

2 *Suction electrode technique*

This was done essentially as according to the method described by Schütz (1931) (see Fig 15) The suction electrodes consisted of thin polyethylene tube (inner diameter 0 4 mm) and this had a polyethylene

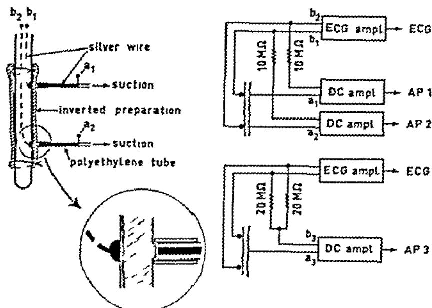


Fig 15 The suction electrode technique The left part of the figure shows the details of the electrode tip and the measuring sites On the right is a semi schematic drawing of the circuit

collar which projected a short distance and which served to give a tighter seal with sucking The electrode lead was a silver wire (diameter 0 1 mm) which passed inside to the end of the narrow polyethylene tube (a_1 and a_2 in Fig 15) The Grass DC preamplifier 5P1 (time constant 0 2 sec) and Grass Model 5 Polygraph Pen Recorder were used for recording The slow frequency characteristic of the latter however made a faithful reproduction of the action potential impossible Since however in most cases it was only the duration of the action potential that was of interest the recorder was satisfactory for this purpose

d) *Weighing*

Analysis tubes and samples were weighed on an analysis balance with an accuracy of 0 1 mg

B Chemical analyses

a) *Sugar determinations with the anthrone method*

Dreywood's anthrone method (DREYWOOD 1946) was used 5 ml of the anthrone reagent were added to 0 5 ml of the sample (about 100 micrograms per ml) (MARSDEN and ÖSTLING personal communication)

After thorough mixing the tubes were warmed in boiling water for exactly 10 minutes and then cooled immediately in running cold water. The absorbance at 620 mμ was then measured with a Coleman Model 4 Spectrophotometer. In 10 determinations of the same sample the standard deviation was ±1.81%.

The blank solution (chapter V A b and c) contained very little anthrone positive substance (corresponding to 0–8 μg hexose).

C. Determination of radioactive isotopes

a) *Gamma spectrometry*

The gamma spectrometer used in both influx and efflux experiments had a 1½" NaI(Tl) well type crystal. A photo multiplier (type RCA 6655) was found not to alter its amplification when the impulse frequency was changed (Öbrink and Ulfendahl 1959). The high voltage supply (Tracerlab Precision High Voltage Supply) had a long term stability of 0.02% per 24 hours or better. The linear amplifier (Tracerlab RLA 1) had good non over load properties (complete recovery after a pulse 100 times too large was obtained within 6–10 microsec). Two pulse height analysers (Tracerlab RLA 5) were used. The input voltage to the spectrometer was provided from a stabiliser (228 V ±0.2%).

The efficiency of this spectrometer system was about 10% with the window wide open.

The energy spectrum for the isotopes used was recorded as according to the method described by Öbrink and Ulfendahl (1959).

Both influx and efflux experiments make great demands on the gamma spectrometer. In an influx experiment the activity during a measuring period is very high and in addition the radioactivity between the measuring periods is between 100 times and 1000 times greater than during the measuring period itself. During the influx experiments the radioactive analysis began only about 50 milliseconds after the radioactivity in the well crystal had been very high (see Fig. 23 p. 47). For such analyses a very good long and short term stability is required and also a linear response up to about 10 000 cps. Further the amplification must be constant and completely independent of the incoming pulse frequency. It was not permissible for the apparatus to have either a short or long term memory.

Analysis of a dilution series of samples of Cs^{137} (Fig. 16) with a wide open energy channel gives a good example of the linearity of the apparatus for incoming pulses (analysis in both energy channels gave the same result but only one of these is shown in the figure). As is evident from Fig. 16 the coincidence losses between 0 and 10 000 cps were negligible.

Fig. 17 shows the gamma energy spectrum for Cs^{137} (sample 2 in Fig. 16) which was obtained by scanning with an energy channel of 0.004 MeV. By analysing a series of dilutions (the same series as in Fig. 16)

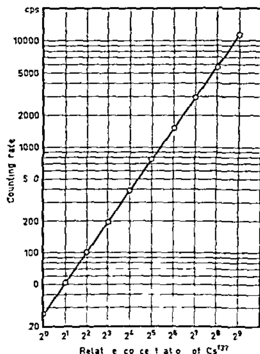


Fig 16 Analysis of a dilution series of Cs^{137} (channel width 0–1 2 MeV)

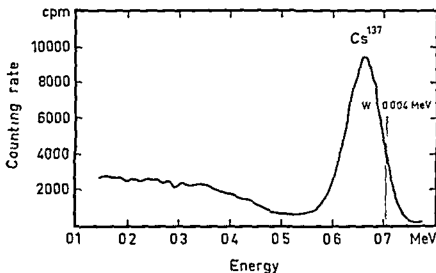


Fig 1" Spectrum of Cs^{137} obtained by scanning with a channel width corresponding to 0 004 MeV (line in figure)

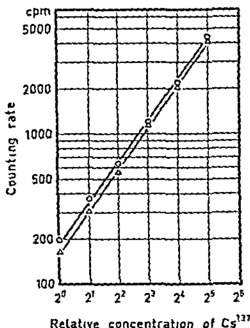


FIG 18 Analysis of a dilution series of Cs^{137} with a narrow window positioned at the steepest part of the Cs^{137} photopeak i e at the line IV in Fig 17 (circles channel I) The other line (triangles) gives the results when the window was set at an energy level slightly higher (channel II) A linear relationship was obtained in both cases

with a narrow window (the 0 004 MeV line in Fig 17) on the steep slope of the Cs^{137} photo peak the results shown in Fig 18 were obtained From these results it can be concluded that the amplification of the apparatus did not change up to an impulse frequency of 10 000 cps (the relative concentration of $Cs^{137} = 2^5$ in Fig 18 corresponds to an input impulse frequency of about 10 000 cps)

The error in radioactive analysis arises from both the statistical error (the radioactive disintegration) and instability of the apparatus The statistical error depends on the number of counts recorded (see for example SCHMEISER 1961) If n is the number of recorded impulses (Poisson distribution) the statistical error in this determination is $\pm\sqrt{n}$ i e ± 1 S D $= \pm\sqrt{n}$ (variation coefficient $v_{stat} = \pm\sqrt{n}/n \times 100\%$)

When a sample of Cs^{137} (2^5 Fig 18) was counted repeatedly with a narrow energy channel situated as in Fig 17 the coefficient of variation for 50 measurements was $v_{m\,(tot)} = \pm 0\ 25\%$ (DANIELSON and SJOSTRAND 1964) This is in fact the same value as the expected v_{stat} corresponding to ± 1 S D and it can therefore be concluded that the stability of the apparatus was very good and that the analysis error for an isotope approached

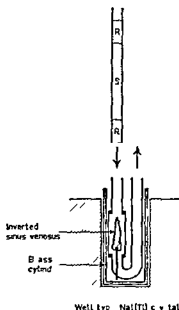


FIG 19 Drawing of an isolated inverted sinus venosus in the U tube which formed the perfusion chamber in an influx experiment (DMM) The whole chamber is mounted in the well of the NaI(Tl) crystal The alternate I inger (R) and silicone oil (S) periods are shown diagrammatically above

that dependent on the variations in the radioactive disintegration process alone In the influx experiments a broad energy channel was always used (wide open window) and it can therefore be estimated that the error on analysis of a single isotope was approximately $\pm\sqrt{n}$

1) *Determination of Na* ^{4} *K*^{4} *and Br*^{8} *in influx experiments the direct monitoring method (DMM)*

In the influx experiments the perfu ion chamber (see chapter III) was placed in the well of the crystal as shown in Fig 19 so that good geometrical conditions were obtained The crystal was mounted in a lead collimator For the analysis of Na ^{4} and K^{42} the perfusion chamber was surrounded by a brass sheath (3000 mg/cm^{2}) For the analysis of Br the brass sheath was replaced by one of perspex with the same dimensions During the influx experiments therefore the preparation always occupied the same place in the well crystal so that identical geometrical conditions were obtained The brass sheath was calculated to absorb all beta particles with an energy lower than 4 0 MeV without giving any significant Brehmsstrahlung Similarly it was calculated that the beta particles from Br^{82} should be stopped by the perspex sheath and the aluminium housing of the crystal The preparation its lf was mm from the

Fig 20 Block diagram of the recording system used in an influx experiment with the direct monitoring method (DMM) For further details see text

bottom of the well in which position the relative counting rate was 97–94 % of the maximal obtainable if the tracer had been placed in the bottom of the well

The radioactivity in the influx experiments was measured in two ways as shown in the block diagram of Fig 20 The pulses from one of the pulse height analysers (channel I) were fed into a decimal pip unit (DANIELSON ÖBRINK SJÖSTRAND and WALLER 1963) The pulses from the decimal pip unit were fed into one channel of a two channel oscilloscope (Tektronix type 502 Dual Beam) and the oscilloscope screen was photographed continually A decimal pip recording is shown in Fig 23 (p 47) The film speed was known and therefore the radioactive counting rate during short time periods could be calculated with great accuracy

This decimal pip method is however rather time consuming and another less laborious method was used for the analysis of the silicone oil perfusion periods during the long influx experiments A digital recording method as shown diagrammatically in Fig 20 was used instead (PERSSON and SJÖSTRAND 1964) Impulses from the second pulse height analyser (channel II) were fed into a common input for two electronic scalers (scalers A and B in the figure)[1] Scaler A or scaler B can be read alternately by a digital printer which can make three readings in one second The programming unit which controls the two way valve which gives the alternate Ringer silicone oil flow in the perfusion system in the influx studies controls also scalers A and B and the reading of these scalers by the digital printer A monitoring pulse (DC) indicating when scalers A and B are counting is recorded on a separate channel of the Grass Model 5 Polygraph (also used for continual ECG recording) and serves

[1] Transistorized scalers with Decade Counter Tubes type ELESTA EZ 10 B see ELESTA technical communications No 18 April 1962 ELESTA electronics Ltd Switzerland

[2] Kienzle Digital Printer type D1E Kienzle Apparate GmbH West Germany

TABLE 3 *Simultaneous determination of radioactivity with both decimal pip and digital printer systems*

Monitoring time (t_m) msec	Counts recorded by printer system	Counts recorded by decimal pip system	Cps according to printer system	Cps according to decimal pip system
1315	320	320	243	243
1230	310	310	252	252
1225	343	330	280	269
1200	317	310	264	258
1185	272	270	230	228
1160	288	280	248	241
1175	306	310	260	264
1160	311	310	268	267
1160	269	260	232	224
1160	301	300	259	259
1125	357	350	317	311
1130	288	280	255	248
1140	307	300	269	263
1140	263	260	231	228
1120	306	310	273	277
1130	283	280	250	248
1140	254	250	223	219
			Mean 256	Mean 253

Simultaneous determination of radioactivity with both analysis systems of Fig 20 (decimal pip and digital printer systems) The monitoring time (t_m) was the same for both systems The values shown correspond to the first 17 analysis points in the experiment illustrated in Fig 27

to indicate the analysis time (paper speed 100 mm/sec see Fig 23 p 47) After each scaler has been read off by the digital printer it is immediately re set to zero The two scalers work alternately while one is being read off the other is counting The mean counting rate during the whole analysis period can thus be estimated

The monitoring pulse was used to operate the digital printer while camera recording of the decimal pip traces was made continuously It seemed possible that the start and stop counting used by the digital printer system might give variations in the calculated counting rate since the start and stop method might not be electronically stable Table 3 illustrates a comparison between the results obtained with simultaneous determination of the counting rate by the decimal pip and direct digital printer systems As is evident from the table any differences are insignificant The values shown in the table are t[illegible] n an experiment which is illustrated in Fig 27

In order to estimate the error of the method for the two analysis systems the dilution series of Cs^{137} used in the experiment illustrated in Fig 16 (p 36) were used (channels I and II 0–1 2 MeV) The analysis periods were 1000 msec long and the duration could be accurately determined both from the Grass recorder and from the photographic recording (paper and film speeds 100 mm/sec) together with the monitoring pulse signals (50 analyses per min) 30–40 determinations were made for each sample of the dilution series Up to a counting rate of about 10 000 cps the same coefficient of variation as expected from the random character of the radioactive disintegrations was obtained both for the decimal pip and digital printer recording i e the coefficient of variation $t_{stat} = t_{method} = \pm\sqrt{n}/n \times 100\%$

Specific activity is defined as Curies per g of the element In this paper however the radioactivity per equivalent concentration i e kcps/µmole will be used and this will be denoted as the equivalent specific activity (ESA) (kcps = 10^3 cps)

2 *Determination of Na^{24} and Br^{82} in mixtures*

The determination of the isotopes in such a mixture was performed as described by ÖBRINK and ULFENDAHL (1959) To minimize the error wide energy channels were used so that a large number of impulses could be obtained in a short time By selecting the channel boundaries in suitable parts of the gamma energy spectrum it is possible to minimize effects of instability of the apparatus (cf DANIELSON and SJOSTRAND 1964) For the analysis of Na^{24} and Br^{82} the channel settings were such that in channel I all energies below 4 0 MeV and in channel II energies between 1 0 and 4 0 MeV were recorded The results shown are in all cases the activities calculated for channel I By frequent quotient determinations (each 5th sample was a standard) any drifts in the channels could be corrected The accuracy of analysis in the determination of Na^{24} and Br^{82} is given for each experiment (see DANIELSON and SJOSTRAND 1964) In no sample did the volume exceed that which gave less than 99–100% relative counting activity In this way the recovery was constant for both isotopes In all cases a correction for radioactive decay during the analysis time was made The values given for the equivalent specific activities (ESA) for both isotopes in the soaking solution are corrected for decay to a constant starting time

b) *Determination of S^{35} by liquid scintillation counting*

The determination of S^{35} was done in a Packard Liquid Scintillation Counter type TRI CARB model 314 EX

0 1 ml of sample (aqueous solution) was added to 3 ml of absolute alcohol in a plastic counting vial 10 ml of scintillation fluid (1 litre toluol 5 g PPO 0 3 g dimethyl POPOP as described by HAYES 1962) were added Since the alcohol and scintillation fluid were added with automatic syringe pipettes any errors so produced must have been very small

D Special methods

a) *Determination of the velocity of flow in the perfusion system*

1) *Determination of the linear mean velocity of flow for continual Ringer or silicone oil perfusion*

In these measurements the perfusion system and perfusion chamber were set up as described for the influx experiments (chapter III) The perfusion pressure was about 735 mm Hg (over pressure) (Fig 11 p 28) The volume of perfusant passing the perfusion chamber in a minute was determined and from the data of the perfusion chamber's dimensions the velocity could be calculated The velocities shown in Fig 14 (p 31) are for Ringer and silicone oil flows calculated for the incoming tube (diameter 1 7 mm) to that part of the perfusion chamber where the preparation was mounted

2) *Determination of the Ringer silicone oil phase boundary velocity*

The Ringer silicone phase boundary can be described as an acceleration–deceleration system and it would therefore be expected to have a lower speed than either Ringer or silicone oil alone The Ringer solution is a volume conductor while silicone oil can be regarded as an insulator The system shown in Fig 21 was therefore used for determining the velocity of the passage of the phase boundary across the perfusion chamber

Points *FW 1* and *FW 2* are equidistant from the centre of the preparation in the perfusion chamber At each point a pair of silver wires (distance apart 2 mm) were inserted [1] The resistance between the silver wires at points *FW 1* and *FW 2* was different for Ringer and silicone oil This was recorded through a Grass 5P1 DC preamplifier and Grass Model 5 Polygraph Since the distance between *FW 1* and *FW 2* (100 mm) was known the velocity of the Ringer silicone oil phase boundary could be calculated

b) *Determination of the conduction velocity of the preparation*

The method is illustrated in Fig 15 (see chapter IV A c 2 suction electrode technique) This method allowed recording of action potentials in positions b_1 and b_2 The preparation was stimulated at the upper end with a rectangular pulse between two silver silver chloride electrodes (not drawn in the figure) The time difference between the action potentials recorded at the two sites AP 1 and AP 2 respectively could be determined Assuming linear propagation in the preparation the minimal conduction velocity for the distance b_1–b_2 (6 0 mm) could be determined

[1] The distance between *outlet S or R* in the rapidly acting two way valve (Fig 12 p 29) and *FW 1* was 85 mm the distance between *FW 2* and the *air inlet* (Figs 11 22 p 28 and p 46 resp) was 160 mm

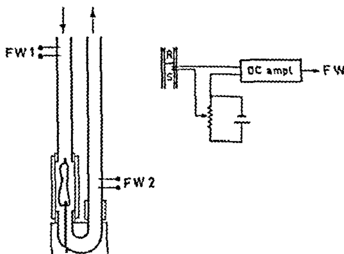


FIG 21 System used for determination of the linear velocity of the Ringer silicone oil phase boundary between points *FW 1* and *FW 2* which are equidistant from the tissue preparation See text for further details

E Solutions

Solution I (*normal Ringer solution*) 6 5 g NaCl (111 2 mM) 0 2 g KCl (2 68 mM) 0 2 g $CaCl_2$ (1 80 mM) 0 1 g $NaHCO_3$ (1 19 mM) and 1 g glucose (5 55 mM) per litre H_2O (pH ≈ 7 [illegible])

Solution II In the experiments where Br^- were studied part of the NaCl was substituted by NaBr This Ringer solution contained 4 8 g NaCl (83 0 mM) 2 9 g NaBr (28 2 mM) 0 2 g KCl (2 68 mM) 0 2 g $CaCl_2$ (1 80 mM) 0 1 g $NaHCO_3$ (1 19 mM) and 1 g glucose (5 55 mM) per litre H_2O (pH corrected to 7 2)

Solution III In the experiments where the different spaces of the preparation were studied the test substance was added to either solution I or II (without glucose)

a) 5% dextran Ringer (R_D) solution I without glucose containing 50 g of dextran 250 per litre ($\bar{M}_W$ = 267 000)
b) 5% ficoll Ringer (R_F) solution I without glucose containing 50 g of ficoll per litre ($\bar{M}_W$ ≈ 100 000)
c) 2% raffinose Ringer (R_R) solutions I or II without glucose containing 20 g (34 mM) of raffinose per litre (M = 594)
d) 2 5% sucrose Ringer (R_S) solution I without glucose containing 25 g (73 mM) of sucrose per litre (M = 342)

Solution IV In the time lag studies a Ringer solution containing 44 mM K^+ per litre was obtained by substituting NaCl in solution I with equimolar quantities of KCl

Solution I In the efflux experiments made with $S^{35}O_4$ a Ringer solution which also contained SO_4 was used This Ringer solution contained 5 7 g NaCl (97 5 mM) 1 4 g NaBr (13 6 mM) 0 2 g KCl (2 68 mM) 0 2 g $CaCl_2$ (1 80 mM) 0 1 g $NaHCO_3$ (1 19 mM) 0 1 g Na_2SO_4 (1 00 mM) and 1 g glucose (5 55 mM) per litre H_2O (pH corrected to 7 2)

No correction was made for the colloid osmotic pressure in the Ringer solutions either for the absence of plasma proteins or the presence of high polymers

The pH of the Ringer solutions was always corrected to pH = 7 2 as there is presumably a marked pH effect on the electrolyte distribution (for K^+ and Cl^- in skeletal muscle cf BROOKS and HUTTER 1964)

Radioactive Ringer solution was obtained by adding $Na^{24}Cl$ $K^{42}Cl$ NH_4Br^{8} and $Na_2S^{35}O_4$ to the solution Na^{24} and K^{42} were obtained by neutron irradiation of $NaHCO_3$ and K_2CO_3 which were then converted to the chlorides by titrating with HCl Br^{8} was obtained by neutron irradiation of NH_4Br and dissolved after irradiation in distilled water [1] When the irradiated material was added the Ringer solutions were corrected so that the molarities of the radioactive solutions I II III and V had the same values as given above S^{35} was obtained in the form of carrier free $Na_2S^{35}O_4$ in aqueous solution [2] The calculated equivalent specific activity (ESA) is given in each experiment

Both the Ringer solution and silicone oil were oxygenated before addition to the perfusion system (without CO_2)

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[1] The isotopes were obtained from AB Atomenergi Stockholm Sweden

[2] The isotopes were obtained from The Radiochemical Centre Amersham England

V Procedure

A Influx methods

Two types of influx methods were used In one the preparation was perfused with an alternate Ringer-silicone oil flow with analysis of the preparation's radioactivity during the silicone oil period (see chapter III for the perfusion system and chapter IV for the direct monitoring method) The second influx method was more conventional (see for example WILDE 1945) The preparation was suspended in a loading solution containing a test substance with vibration stirring and after a certain time transferred to a bath of inactive Ringer solution From the analysis of the washing solution the amount of test substance taken up could be calculated

a) *Direct monitoring method (DMM)*

After the preparation had been mounted in the perfusion chamber (see Fig 22 direct monitoring method) it was perfused with inactive Ringer solution for about 30 min (see preparation of the toad heart p 16) the ECG being recorded continuously during this period If the preparation had a constant spontaneous frequency and the form of the ECG remained unaltered during this 30 minute period the experiment was then continued if the position of the preparation had remained satisfactory during the preliminary perfusion The programming unit was now adjusted so that counting (start and stop order to the digital printer system see Fig 20 p 39) only took place while the preparation lay in the silicone oil phase This was accomplished with the two pairs of resistance electrodes which had been used to determine the velocity of the silicone Ringer phase boundary (chapter IV D a 2) In each experiment the length of the Ringer perfusion period is given as t (usually about 100 msec) that of the silicone oil perfusion t (≈ 1200 msec) and the duration of the radioactive measuring period (monitoring time) t_m (≈ 1000 msec) The next stage of the experiment was the replacement of those parts of the U tube

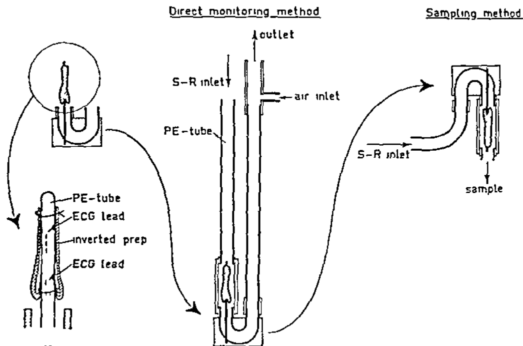


FIG 2. The arrangements of the perfusion chamber used for the direct monitoring (DMM) and sampling (SM) methods On the left is shown an inverted sinus venosus mounted on a polyethylene ECG catheter (*PE* tube) attached to the L tube This is the unit which was used for both influx (DMM) and efflux (SM) studies *S–R* inlet = silicone Ringer inlet The *air inlet* corresponds to that shown in Fig 11 (p 28)

system which contained the silver resistance electrodes by polyethylene tubes It was now first necessary to wash out the inactive Ringer solution from the tube leading to the preparation chamber so that the influx study should begin with perfusion by a solution of specific activity the same as that in the vessel *Ra* The preparation was therefore perfused with silicone oil at a low velocity (low driving pressure) while the two way valve in the *Ri Ra system* (see Fig 11 p 28) was switched to active Thus active Ringer passes through to the rapid two way valve and out through the *bypass outlet R* (Fig 12 p 29) The activity of the solution was monitored with a beta detector and a sample taken for later accurate determination of the activity per unit volume The *bypass outlet P* was then closed and the driving unit to the rapid two way valve (see Fig 20 p 39) disconnected while the background activity was determined with the digital printer system During the following silicone oil perfusion the perfusion

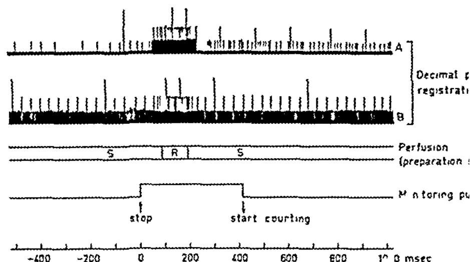


FIG 23 Decimal pip recording from two different periods in the influx experiment illustrated in Fig 3? (p II) *A* is the record of the oscilloscope screen showing the first active Ringer perfusion period (*r*1) *B* includes the record of the 76th active Ringer period (*r*76 in Fig 3, curve I $t - t_s = 143$ sec) The relative length of the active Ringer period (*R*) is shown schematically flanked by silicone periods (*S*) It can be seen that this continuous recording reveals that the phase of increased activity precedes and lags behind a little the actual region of the active Ringer solution As is shown the monitoring pulse stops recording by the digital printer both during the active Ringer period and immediately before and after it

pressure was raised to about 73υ mm Hg (over pressure) and then the alternate perfusion with radioactive Ringer solution together with analysis of the activity during the silicone oil period was started ECG and the monitoring pulse were recorded on the Grass recorder while the decimal pip recording and monitoring pulse were photographed on the oscilloscope screen (cf Fig 23) which as a rule was only filmed during certain parts of the experiment At the end of the experiment a sample of the active Ringer solution was taken via *bypass outlet R*

b) *Soaking experiments with vibration stirring*

The preparation was mounted as described on the ECG catheter and was mounted in the perfusion chamber as for influx experiments It was perfused for 30 min with inactive Ringer solution after which

the preparation was perfused with silicone oil at full perfusion pressure ($\bar{v} \approx 300$ cm/sec) for 3 sec The ECG catheter together with the preparation was then removed from the perfusion chamber and mounted on a stand It was then dipped in active Ringer solution (active in this sense means that the Ringer solution contained the test molecules or isotopes) The vessel containing the active solution had a volume of 1 ml and was attached to the vibrating shank of a small magnetic vibrator The active solution was oxygenated immediately before addition to the tube, and in those cases where the preparation was soaked for longer than 5 minutes this was exchanged for freshly oxygenated Ringer solution each fifth minute After a certain time the preparation was then quickly remounted for perfusion with silicone oil and perfused at a mean velocity of about 300 cm/sec for 3 seconds This short silicone oil period washed away the greater part of the Ringer solution which had been carried over with the preparation which was then again removed from the chamber and washed in small vibration stirred tubes containing inactive Ringer solution The washing time was always at least 1½ times the soaking time but was never less than 10 minutes The volume of inactive solution used in the washing off tubes was about 0 5 ml (the exact volume was determined by weighing) The inactive washing solutions were also oxygenated and changed for new solution every fifth minute The amount of test solute which had accumulated in the preparation was calculated by measuring the concentration in the pooled solution from all the washing tubes used In order to check that all activity had been washed out of a preparation it was in some cases verified that the concentration of the test species was zero in the last washing out solution The treatment in the Ringer baths was at room temperature and the ECG was followed during the whole experiment The washing off phase with silicone oil was performed at +5°C for 3 sec This temperature was used in order to obtain similar surface layers around the preparation as in the influx experiments made with the direct monitoring method Since certain substances normally present in cardiac muscle give a positive anthrone reaction (carbohydrate determination) the blank solution for the anthrone determinations was obtained by washing out the preparation in inactive Ringer solution for 10 minutes subsequent to a 10 minutes soaking period in inactive solution

c) Perfusion experiments with washing-out of the activity in a static solution bath

The preparation was mounted in the chamber as in the direct monitoring method and the procedure up to the washing through with and sampling of the active Ringer solution was the same as described in section a (direct monitoring method) The programming unit (Fig 20 p 39) was then adjusted for the selected Ringer perfusion time The perfusion pressure was raised during the silicone oil perfusion to a value of about 735 mm Hg (over pressure) The Ringer solution was then perfused for the selected period and this was followed by silicone oil perfusion (ι ≈300 cm/sec) for 3 seconds The preparation was then quickly removed from the perfusion chamber and washed in inactive Ringer at room temperature as described under section b When necessary the blank for the anthrone method was also taken in the same way as described previously

B Efflux methods

a) *Sampling method* (SM)

The preparation was mounted on the ECG catheter in the chamber and perfused with inactive Ringer solution for 30 minutes its viability was assessed as described previously under section A a (DMM) The preparation was then removed from the chamber and loaded with the isotope to be studied by soaking for a certain time in an oxygenated and vibration stirred bath of active Ringer solution (room temperature) The solution was renewed each tenth minute The ECG was recorded continuously during the loading period A sample of the last active Ringer solution was taken for analysis The preparation was then returned to the chamber but this time mounted as for an efflux experiment as shown in Fig 22 (p 46 sampling method) It was then washed with silicone oil and the ECG and frequency of spontaneous beating were observed When it was judged from the ECG that the preparation was in thermal equilibrium with the perfusion solution inactive Ringer solution was perfused through the chamber and collected in test tubes mounted in a fraction collector operating at two changes per second Each time the sample tube was changed this was recorded on one of the channels of the Grass Polygraph recorder (paper speed 50 mm/sec)

VI *Some Influx Experiments, the Calculations and the Graphical Representation of Such Experiments*

As discussed previously (chapter IV C a 1 direct monitoring method) the error of the method in the radioactive analysis in the systems used depends almost entirely on the number of impulses recorded Since the monitoring time (t_m) was of the order of 900–1400 milliseconds it was necessary if the counting error was to be small to have a high radioactivity during the period of analysis This means that the radioactive Ringer solution should therefore have a high specific activity In influx studies therefore only isotopes where the decay gave a large percentage of gamma radiation were suitable

The ions which from a biological point of view are of greatest interest are Na^+ K^+ Cl^- and Ca^{++} Na^{24} can be obtained with a very high specific activity and has two gamma peaks (1 37 and 2 76 MeV) both of which are obtained in 100 % of disintegrations In the case of potassium the only suitable isotope available until recently is K^{42} this has a gamma peak at 1 53 MeV but only 18 % of disintegrations give this energy With the irradiation of normal potassium an insufficient specific activity of K^{42} is obtained even with high neutron fluxes By irradiating enriched K^{41} or through the irradiation of normal potassium with very high neutron fluxes the specific activity of K^{42} can be increased but this is very expensive Because therefore of the relatively low specific activity of K^{42} only a few experiments have been made with this isotope (see Fig 31 p 69)

Cl^{36} is only a beta emitter so it is not suitable for an influx experiment Cl^{38} is from many points of view unsuitable It has a short half life and a beta energy of 4 81 MeV (53 %) which makes it somewhat of a health hazard Under the assumption that Br^- and Cl^- are distributed in the same way in heart tissue (LAMB 1961 HUTTER 1961 BURROWS and LAMB 1962) experiments have been made with Br^{82} According to DANIELSON (1964) the Br^- and I^- ions distribute

in the same way as the Cl^- ions in the sinus venosus atrium and ventricle of the toad Br^{82} can be obtained with high specific activity and has eight gamma peaks between 0 55 and 1 47 MeV Of the Ca isotopes Ca^{45} has no gamma emission while Ca^{47} although a gamma emitter was rather too expensive for an influx experiment

An influx experiment with Na^{24}

Fig 24 illustrates an influx experiment with Na^{24} on an isolated sinus venosus The preparation had an ECG which showed that for each heart cycle there were two electrical activities (cf Fig 9 p 24) It was also observed that the preparation had two mechanical activities The upper part contracted just before the lower part Two influx experiments were made on this preparation of which the first is illustrated in the upper part of the figure The radioactivity counted during a silicone oil period has been recorded as though it occurred just at the end of the foregoing Ringer perfusion period The radioactivity counted increased with time during alternate active Ringer and silicone oil perfusion

After the first influx experiment was finished all radioactivity was removed from the preparation by perfusion with inactive Ringer solution for about 30 minutes

A second influx experiment was then made with the same active Ringer solution as previously and the result is illustrated in the lower part of Fig 24 The sinus venosus beat spontaneously for about 26–27 seconds when a much stronger ECG complex than had previously been observed appeared subsequently no further electrical activity was recorded It is evident from the figure that the radioactivity during the first 20 seconds increased at about the same or a little lower rate than in the first experiment After 22–23 seconds the rate of increase with time rapidly lessened The reason for this sudden cardiac arrest is not clear The spontaneous activity however returned when at the end of the experiment the preparation was removed from the chamber and bathed in Ringer solution at room temperature It therefore seems possible that an undue temperature sensitivity may have been responsible for the cessation of spontaneous cardiac activity If the radioactivity recorded is representative of that taken up during an influx experiment by the sinus venosus there thus seems to be a connection between the electrical and/or mechanical activity of the

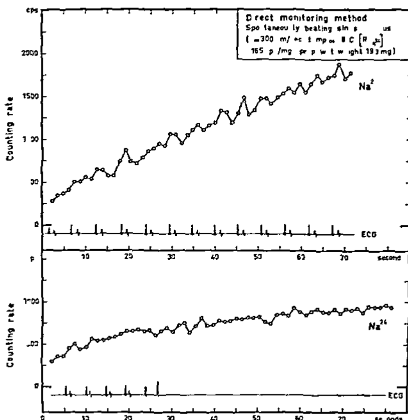


FIG 24 Two influx experiments with Na^{24} on an inverted spontaneously beating sinus venosus perfused alternately with active Ringer solution and silicone oil The radioactivity was measured during the later period (DMM) The ECG showed that for each heart cycle there was a double electrical event (see text) *The radioactivities recorded have been arbitrarily assigned to the time point of the end of the preceding Ringer perfusion period* The upper part of the figure shows the first influx experiment and the lower part the second experiment (about 30 minutes after the first) during which the electrical activity ceased The surface film (O_R) was calculated as equivalent to 1 64 mg of active Ringer solution and the trapped volume as 0 66 mg t = 105 msec t_s = 119 msec t_m = 928 msec and t_{eq} = 400 msec The mean cycle calculated for the first experiment is shown in Fig 47 (p 117)

preparation and the rate of uptake of Na^{24} HAAS GLITSCH and TRAUTWEIN (1963) have shown that Na^{24} efflux from an isolated auricle of the frog is greater in a beating than in a non beating preparation (cf introduction)

In influx experiment with Br^{8}

Such an experiment is illustrated in Fig 25 (p I) where it is evident from the electrocardiogram that the sinus venosus beat with a very

regular frequency during the whole experiment The electrical activity appeared to arise from a single pace maker (FCC) and it was observed visually that the mechanical activity in each cycle was single The radioactivity recorded during the monitoring period (silicone *oil* perfusion) has been plotted as previously as though it occurred at the end of the foregoing Ringer perfusion period It is evident that the radioactivity *increased* with time during the experiment

Trends in the data or only background noise?

It is evident from the Na⁺ experiment and even more so from the Br⁸ experiment that if there are any real variations associated with single cardiac cycles they are confused by other variations such as random fluctuation noise in the perfusion system statistical counting errors and instabilities in the recording system It cannot therefore be expected that radioactivity recorded from each single cardiac cycle will give precisely the same picture The analysis period used i e 1 2–1 3 sec is relatively long in relation to the biological process being studied Thus the influx method from this viewpoint can be regarded as having a rather low resolution In order to ascertain the underlying biological pattern it is therefore desirable to calculate the variations in counting activity for a *mean cycle*

Continuous averaging is a problem which has been treated *inter alia* in connection with the analysis of the electroencephalogram For this purpose a number of different mathematical and graphical methods or harmonic Fourier analysis have been used More recently computers often called CATs (computers of average transients) have been used for solving these problems Their task has been to calculate the mean value of or summate responses as they occur in real time and to accumulate this information The CAT technique has been reviewed by GARRY (1961) The CAT is however not directly applicable to these experiments and for this reason a graphical method has been used to obtain the mean cycle This method will be called the *computing of average transients* *c-a t*

Essential requirements for c a t

The primary assumption in a *c-a t* is that the biological activity curve is a continuous function (a *c-a t* is in fact a smoothing or curve fitting procedure for the oscillatory component) and that the obser

vations are frequent and of short duration compared to the biological oscillations The radioactivity measurements in the actual inflow experiments are of relatively long duration and hence few in number in each cycle so that it may appear questionable as to whether they can be used to deduce a continuous type of biological activity curve

In the analysis described here the number of observations per cycle is small (in Fig 25 ≈ 7) and therefore *the observations from a number of cycles are used to derive the probable activity curve for an 'average single cycle* There is therefore the added absolute requirement that during the actual influx experiment the relationship between the *mean influx* during the Ringer and silicone periods must remain constant for the whole experiment It is assumed of course that the biological processes are essentially the same in each cycle Further variations in the perfusion or analysis systems must be of a purely random nature

The c a t procedure in a Br^{82} influx experiment

The experiment illustrated in Fig 25 serves as an example of the determination of the *mean cycle* by the *c a t* procedure in an influx experiment As is evident from Figs 24 (p 52) and 25 (p I) *the* Na^{24} and Br^{82} *activities appear to consist of at least two components*

1 an exponential influx curve (*DC component*) on which are superimposed

2 oscillations (*AC component*)

1 *DC component* It will be shown in chapter VII that the requirements stated above for the *c a t* are approximately valid

The first stage is to establish a *mean trend* for the increase in activity as a reference or *base line* Even if the biological significance of this line is not established with certainty it seems justifiable for mathematical reasons as a seemingly suitable regression line for all the points The equation for the line which best fits the observations will be called the *mean trend* and can be derived with a curve fitting procedure as described by LEWIS (1960) The general form of the equation is first assumed with the influx experiments it is of the general form $y_t = a(1 - e^{-kt}) + y_{t\,0}$ where y_t is the recorded activity in cps at time t seconds after the beginning of the experiment and a and k are constants

Fig 25 (p I) shows the mean activity calculated for each heart cycle (solid circles) and it can be seen that they lie scattered closely about the curve (calculated from all the points—empty triangles) For the *c-a t* procedure in the other influx experiments the *mean trend* base line has therefore been constructed in a simpler way from the mean values for each cycle rather than from all the individual measurements The lines so obtained are all loci of the type of exponential function given above

2 *1C component* The single heart cycle is arbitrarily defined as beginning 2 seconds before depolarization and is in the experiment Fig 25 7 4 seconds long i e taking depolarization as $t=0$ from -2 to $+5\ 4$ seconds This is illustrated in Fig 26 which is an analysis of the results of the experiment of Fig 25 the figure also shows the first half of the next cycle which is of course merely a repetition of the first cycle *All radioactive measurements are expressed as deviations from the mean trend line and their temporal position in the cycle was determined graphically* (as mentioned previously each measurement is treated as though it occurred instantaneously at the end of the Ringer period preceding the silicone period during which the measurement was made)

The distribution of the radioactive measurements at different time intervals within each heart cycle is relatively uneven [1] *The cardiac cycle was arbitrarily divided into 200 msec intervals and the radio activity measurements were classified graphically into these classes* which are designated by their central values The numbers in each class varied randomly from 4 to 17 the arithmetic mean being 10 6 Thus the number of observations (N) in class $t=400$ msec (301–500 msec) was much less than in class $t=1400$ msec (1301–1500 msec) When N is small such a point is statistically less reliable than with a large value of N and *a curve drawn by interpolating such values is likely to be more uncertain in some intervals than in others* Both random noise (instability) and statistical counting error are very important

[1] The measurements were made at regular time intervals and the heart beat with a relatively constant frequency There were thus two systems with regular frequencies Under certain conditions such systems can exhibit a constant phase pattern with respect to each other i e behave as though they were locked This would mean that the measurements were repeatedly confined to the same parts of the successive cardiac cycles This was not observed in these experiment however

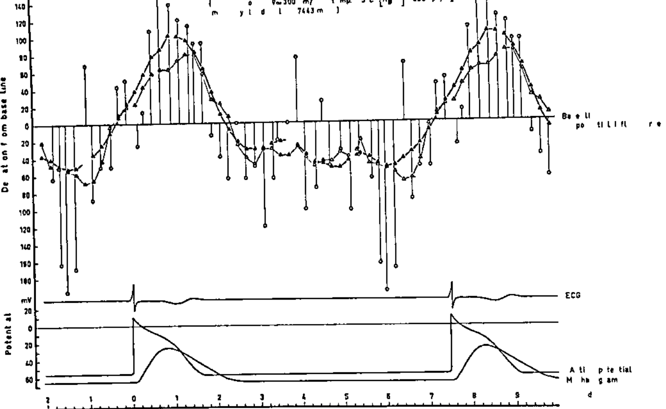


FIG 26 A mean cycle for Br^{82} calculated by the c a t method for the experiment of Fig 25 The base line corresponds to the mean trend line in Fig 25 (curve I) The ECG was recorded in the experiment while the action potential and mechanogram traces are drawn from other experiments of the same type where they and the ECG were recorded (chapter II c) The circles represent the median values for the measured values classified into 200 msec intervals (original value method) The open triangles are the medians and the solid triangles the arithmetic means of 67 interpolation values for each 200 msec interval (intercept–interpolation method) The standard deviations calculated for the original value method and the arithmetic means of the interpolation method are given on p 57

The maximal error of the mean radioactivity curve with respect to time (i e to electromechanical cycle) is $(t + t) = (100 + 1104) =$ 1 04 msec to the left (cf p 83 and p 120) The wet weight of the preparation was 16 1 mg O_R was calculated as 1 4 mg and the trapped volume as 0 7 mg Perfusion and analysis programme (DMM) $t = 100$ msec $t = 1104$ msec $t_m = 930$ msec Active Ringer with 1 8 × 10 5 k ... Br /μ ... Br (solution II) (key a = 10 c/s)

here It seems therefore better to use another type of interpolation method *In each heart cycle the points of observed radioactivity have been joined by straight lines and the values of the intercepts of these lines with the central t class values which they cross have been recorded* Thus for each t class there are as many intercept values as cardiac cycles measured during the experiment (67 in the experiment of Fig 26) independently of how many actual radioactive measurements fell within that t class interval There was of course quite wide variation in the intercept values for any particular class but extreme variations showed a tendency to cancel out each other The result should perhaps be a more certain assessment of the average value for each t value than with mere interpolation from the mean values of the observations themselves

An important question is therefore as to how the observed values of the radioactivity should be treated i e classified into time intervals and the mean class values used directly or subjected to a more complicated interpolation procedure a described above Further in taking averages should the arithmetic mean or the median be used?

Fig 26 shows three out of the four possible methods The *original value method* (open circles and vertical lines) these are the median activities in each t class (200 msec) The results of the *intercept-interpolation method* are also illustrated in the figure the averages of 67 intercept values in each 200 msec interval are calculated from the *arithmetic means* (solid triangles) and the *medians* (open triangles) As is evident from the figure all three methods give essentially similar pictures The area enclosed by the curve above the line is also approximately equal to that below the line which should be expected when a mean cycle about a base line is determined

The points of the *original value method* in Fig 26 are the median values of the observations from all the heart cycles classified into 200 msec intervals The number of values in each class interval ranged from 4 to 17 It is evident that the average values exhibit irregular and large positive and negative deviations although the general trend is clear The standard deviation of the mean in the intervals with the greatest number of observations (8 intervals had 14–17 observations) was on the average ± 7 7 (cps) The corresponding value for the same eight intervals in the *intercept interpolation method* (67 points in each case) was ± 6 5 The difference is not significant (i e with $\Lambda = 16$ and $\Lambda = 67$ respectively it is not even

as great as the mean error of the difference) but with a lower value of N extreme values would have more influence With a standard deviation of about 7 0 the mean error with for example only 6 observations ($N=6$) will become more important It is therefore not surprising that a direct use of the average of the observations in each class interval (original value method) gives a much less smooth result than with intercept–interpolation method The latter may therefore give a clearer picture of the activity pattern

Fig 47 (p 117) *shows a similar treatment of the results of a Na*[+] *influx experiment* Only median values were used for both the original value and intercept–interpolation methods which show fairly good agreement if allowance is made for the irregularities of the former method In Fig 48 (p 118) which illustrates another experiment the arithmetic means have been used in calculating the points by the two methods *From these results it is evident that in calculating the mean cycle the interpolation method is more satisfactory It must however be considered which average should be used the median or arithmetic mean* In general the median is more suitable if N is small and extreme values are more likely to be disturbing and produce asymmetric distributions If there are no or very few asymmetric extreme values the arithmetic mean can equally well be used even with a small number of observations Since it seems reasonable to presume that the biological flux pattern is a continuous funtion of time this means that *large variations in the recorded radioactivity can be expected to result from such factors as the length of the analysis period in relation to the period of the biological curve and the temporal position of the analysis period in relation to the biological events*

Assuming that this argument is correct the calculation of *mean values from the interpolated values should be used only if N is relatively large* Only then can it be assumed that extreme values resulting from random noise in the system will be cancelled out Biological extreme values should then be revealed by the averaging of the intercept–interpolations *When N is small the median should be used* in order to minimize the effect of extreme values which may be of random noise character

It should be further pointed out that the intercept–interpolation method does itself modify the pattern of radioactivity The amplitude of fluctuations from the base line will be reduced somewhat but the variations around the mean will not be essentially altered (cf Figs 26 47

VII *Some Methodological Problems of the Influx Experiments*

Some of the most important problems associated with the results discussed in chapter VI must now be appraised

A The magnitude of the extraneous or background activity is important in the influx experiments This activity is partly due to imperfect shielding from active Ringer solution in the incoming and return perfusion lines A further source of activity extraneous to the tissue preparation is the trapped activity in the perfusion chamber itself this was measured in a sham experiment without any tissue in the chamber

B For physical reasons it can be expected that the tissue preparation will be surrounded by a thin surface film of Ringer solution during the silicone oil perfusion period The thickness of this layer and whether it remains constant during one silicone oil period and from period to period are important

C If there is a significant surface layer whether the rates of increase of radioactivity in the tissue during Ringer and silicone oil perfusion are the same During Ringer perfusion the radioactivity source is virtually infinite while in the silicone period it is finite and not very large The increases therefore during the two periods must be expected to be *different functions* of time but it is important that their *mean values* have a constant ratio during the whole experiment (when the perfusion intervals are held constant)

D The volume(s) in the tissue preparation which are most nearly equilibrated with radioactivity during the experiment

E The time lag between contact with the perfusing solution (Ringer) and entry of radioactivity into the tissue with the DMM (transport across a surface layer)

F The effect of the mechanical contraction on the surface layer (or extracellular space)

Experimentally it is simpler to approach problems B and C with an object whose structure is much better defined than in a biological preparation (e g cellophane or an ion exchanger) As the biological problem in the present experiments has been the primary aim it seemed more important to attempt to appraise these matters in relation to the actual heart preparation used as they are very important in the analysis and interpretation of the results of both influx and efflux experiments A more systematic study of for example an ion exchanger has not yet been made but seems desirable

The methods used for studying problems A–F will be described below and *the most important methodological problems concerning the results will be discussed the question of their biological significance will be deferred to general discussion* Point F will be mentioned very briefly

A The background activity and "trapped activity"

Sham "influx" experiments for Na^{24} and Br^{8} were made with an empty perfusion chamber in all other respects the technique was as described in chapter V A a (direct monitoring method) The result for a sham Na^{24} "influx" experiment is shown in Fig 27 where the background was determined before the start of an alternate Ringer silicone oil perfusion *(t_r = 100 4 msec t_s = 1320 msec t_m = 1160 msec* cf definition in chapter V A a) It can be seen that after the first active Ringer period the radioactivity lies around a level which subsequently remained constant at a mean value of 60 cps above background From the known counting activity of the active Ringer solution (cps/μl 1 μl ≈ 1 mg) the trapped activity can be estimated as equivalent to 0 42 mg of the active Ringer solution The line best fitting the observations was calculated by BARTLETT's (1949) method The coefficient of variation was $\varepsilon_{method} = \pm 8\,7\,\%$ Analysis of a Cs^{137} sample in the same analysis system and method (see chapter IV C a 1) gave a coefficient of variation $\varepsilon_{stat} = \pm 5\,9\,\%$ ($\varepsilon_{stat} = \pm \sqrt{n}/n \times 100$) In a similar experiment with Br^{8} *the activity of the Ringer* solution was 174 cps/mg (t_r = 100 4 msec t_s = 1320 msec and t_m = 1001 msec) The trapped activity corresponded to 0 90 mg active Ringer solution ($\varepsilon_{method} = \pm 8\,0\,\%$ and $\varepsilon_{stat} = \pm 5\,3\,\%$) in this experiment also the chamber activity reached a constant level after the first perfu

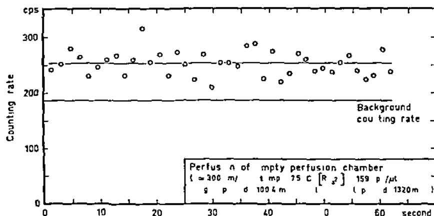


FIG 2 Perfusion of an empty chamber with active Ringer solution (DMM) The first 17 analyses are also shown in Table 3 $\iota_{method} = \pm 8\ 7$ $\iota_{stat} = \pm 5\ 9$ (cf text)

The *trapped volume* will be assumed to be equivalent to a mean value of 0 7 mg Ringer solution in those cases where it was not measured Whether the difference between the Na[4] and Br[8] values is random or not is not known but practically it seems justifiable to use the average value

The *background activity* was 186 cps in the Na^{24} experiment corresponding to 1 17 mg of the active solution and with Br^{8} 215 cps corresponding to 1 21 mg

Summary The background activities in sham influx experiments were equivalent to about 1 2 mg active Ringer solution while the trapped activity remaining in sight of the NaI(Tl) crystal corresponded to 0 7 mg *During sham perfusion with alternate active Ringer solution and silicone oil as described for D M M* (chapter V A a) *the radioactivity varied randomly around a mean value* (cf Fig 27) *where the coefficient of variation* (ι_{method}) *was only a few per cent greater than the statistical counting error* (± 1 S D) *for the mean number of counts recorded* (total counts including background)

B The surface layer of the heart preparation during Ringer and silicone oil perfusion

As mentioned earlier both the Ringer and silicone oil flows are turbulent In the turbulent Ringer flow good contact between the perfusant and the tissue can be expected even if an unstirred layer

cannot be avoided. The thickness of the unstirred layer is difficult to estimate theoretically in the system used (see JOST 1952 p 78) but in physical studies of flows in the vicinity of surfaces BOCK (1963) concluded that "unstirred layers" of the order of 0.5–1.0 μ might exist for Newtonian liquids. COPLEY, SCOTT BLAIR, BALŁA and STAPLE (1960) showed that this order of magnitude also applied to the "plasmatic zone" in blood vessels of the Chinese Hamster. SCHULMAN and TEORELL (1938) and MANEGOLD and KALAUCH (1939) gave values for unstirred layers of the order of 10–30 μ. Since silicone oil is strongly hydrophobic it may be expected that during its passage through the perfusion chamber the tissue preparation will be enveloped by an aqueous surface film (O_R) consisting of Ringer solution notwithstanding the turbulence of the silicone oil stream. There will thus be an interface between the external surface film of aqueous solution and the main silicone oil phase. The surface aqueous phase under these conditions is probably larger than the "unstirred layer" in Ringer perfusion. During silicone perfusion the aqueous surface film may however undergo a certain agitation due to pressure variations resulting from turbulence of the passing silicone oil stream. Such pressure variations may also of course be transmitted to the tissue preparation itself.

The volume of the surface film O_R during silicone oil perfusion

Studies of this parameter require water soluble and non ionic (low affinity for tissue proteins) test solutes. Ideally the test substance should be specific for the surface film, i.e. either excluded from or only very slowly penetrating into the other regions of the tissue. (No such ideal substance exists and tests must be made on different substances with the aim of being able to extrapolate the results for the case of the ideal solute.) For a direct analysis with DMM a substance labelled with a gamma emitting isotope obtainable in high specific activity which does not alter the normal physical properties of the Ringer solution is required. Suitable gamma labelled substances were not available so indirect methods were used instead.

Four non electrolytes were used, all of which can be determined with high accuracy by an anthrone method. These were dextran[1] (M_w 267 000), ficoll (M_w = 100 000), raffinose (M = 594) and sucrose (M = 342). Dextran is uncharged polyglucose and takes the configu

[1] Dextran and ficoll were kindly supplied by AB Pharmacia, Uppsala, Sweden.

ration of a loose random coil in solution (NEELY 1960) Ficoll a poly sucrose is a neutral but more compact molecule in solution its solutions have a much lower viscosity than those of dextran The molecular sizes of both dextran[1] and ficoll are such that during the short soaking time diffusion could not account for any significant penetration of the extracellular space Raffinose and sucrose have often been used to determine extracellular space but according to BOZLER (1959 1961a) sucrose penetrated also the intracellular compartment of the frog sartorius muscle (soaking time = 2 hours) The concentrations used were 5 g % for dextran and ficoll 2 g % for raffinose and 2 5 g % for sucrose (w/v) The concentrations were held as low as practicable in order to reduce the osmotic effects on the tissue In order to compare the figures for all four substances determined with the anthrone method in the experiment of Fig 28 the values for raffinose have been multiplied by 2 5 and those for sucrose by 2 The solubility of dextran ficoll raffinose and sucrose in silicone oil is virtually zero[2] After soaking the tissues were placed in the perfusion chamber and washed with either a 3 or 10 second period of silicone oil (linear velocity 300 cm/sec see chapter V A b) which was saturated with the test substance The preparations were then removed and the amounts of the carbohydrates taken up in the soaking bath were estimated there were no significant differences i e the 3 second silicone period was as effective as the 10 second period

To obtain more reliable determinations with this method it seemed desirable that the amount of substance taken up should be larger so the experiments were carried out on isolated atria of toads

Fig 28 shows an experiment where a spontaneously beating atrium was bathed for different periods in dextran Ringer (R_D) ficoll Ringer (R_F) raffinose Ringer (R_R) or sucrose Ringer (R_S) with vibration *stirring according to the method described in chapter V A b* This is

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[1] The so-called mean Stokes Einstein radius of this dextran fraction is estimated as 70 Å (GROTTE, 1956)

[2] The distribution coefficient for dextran between silicone oil and Ringer solution was determined by shaking together aliquots of 5 % dextran Ringer solution and silicone oil (MS 200 2 cS) for 5 min After the two phases had separated half of the silicone oil phase was removed mixed with an aliquot of normal Ringer solution and shaken for 5 minutes The dextran concentration in this second Ringer solution was then determined by the anthrone method and the distribution coefficient of (Ringer) (silicone oil) was calculated from a duplicate determination as 6[illegible] 1

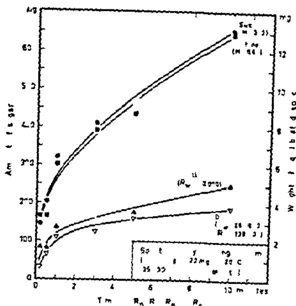


FIG 29 Soaking experiment with vibration stirring as described in chapter V A, b (see text)

one of two experiments both of which showed similar results By assuming that the test molecule equilibrates with an accessible compartment to the same concentration as in the active Ringer solution an *equilibration volume* can be estimated The figure shows the relative rates of penetration of the four substances into the preparation *It appears probable that all four substances equilibrated initially with a space of 2–3 mg in weight A subsequent distribution took place into what appears to be a second compartment* This distribution show a relationship to the decreasing molecular weight (or increasing diffusion coefficient) *It seems reasonable to suppose that the initial equilibration space is a surface film to which all substances have a very rapid access and the second compartment is an extracellular space or even perhaps partially intracellular* (BOZLER 1961*a*)

Fig 29 shows the results of experiments made according to the method described in chapter V A c where the points in the figure are mean values of the results from several preparations (see figure text) *The figure can be regarded as representing to a large extent the experimental conditions during an actual influx experiment by the DMM and shows that equilibration of the surface layer takes place in less than*

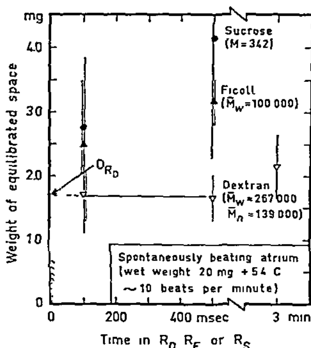


FIG 29 A perfusion experiment with washing out in a static bath on spontane ously beating atria with dextran (R_D) ficoll (R_F) and sucrose (R_S) The proce dure was as described in chapter V A c and the perfusion times 100 msec 500 msec and 3 minutes For R_D 3 atria were used for R_F 4 atria and for R_S . atria The number of measurements at different perfusion times were

100 msec 21 (R_D) 18 (P_F) 9 (P_S)
500 msec 14 (R_D) 21 (P_F) 9 (R_S)
3 minutes 8 (R_D)

The standard errors are drawn in the figure The amount of test substance was corrected in all cases to a preparation wet weight of 20 mg The external surface film (O_{R_D}) was with dextran equivalent to 1 7 mg of the Ringer solution (8 5 %, of the wet weight)

100 msec for all substances (dextran ficoll sucrose) *There was no penetration of dextran into the second compartment during a 500 msec long perfusion period* (cf also the mean values for dextran Ringer solution for a 3 minute period) *Dextran thus seems a suitable indicator substance for the surface layer* at least under the perfusion conditions existing in the influx experiments (DMM $t \approx 100$ msec) *This extra-cardiac surface layer* (*O_{R_D}* in the figure) *during the silicone oil perfusion should therefore for dextran have a volume corresponding to 1 7 mg of Ringer solution (8 5 % of the wet weight)* It seems improbable that any interaction between dextran and the surface proteins of the preparation contributes to this to any significant extent becau e

it is apparent from the figure that the minimum sucrose space is similar (there is no reason to suppose that sucrose should be absorbed to any significant extent) The amount of substance which would accumulate on the preparation assuming a complete monolayer was calculated to be less than the anthrone method error

In order to assess correctly the results shown in Figs 28 and 29 certain other factors must be taken into account The presence of 5% dextran in the Ringer solution greatly increases its viscosity while ficoll raffinose and sucrose in the concentrations used have an insignificant effect on the viscosity (solutions I and II) The colloid osmotic pressure in the dextran and ficoll Ringer solutions can be neglected (Rowe 1955 found that a 4 2% dextran solution was iso osmotic with 7 serum proteins at the concentration and with the molecular weight range of the fraction used here the osmotic pressure is virtually a function of the concentration) The osmotic effects of the oligosaccharides will be treated later (p 87)

In the discussion which follows the *extra cardiac surface film* (O_R) *during silicone oil perfusion* has been assumed to have a size corresponding to that determined with dextran (O_{RD}) i e corresponding to *8 5* *of the wet weight of the preparation*

Unstirred layer during Ringer perfusion

Since the equilibration at the surface during a dextran perfusion seems to require less than 100 msec (cf Fig 29) *it appears reasonable to assume that during the Ringer perfusion periods in the influx experiments (DMM) the surface film* O_R *acquires the same specific activity as that of the active Ringer solution* The minimal surface area of the preparation (the external surface of an open cylinder) was used to calculate that the maximal thickness of the surface film during the silicone oil perfusion ($O_{RD} = 8\ 5$ of the wet weight) was 25–30 μ (the minimal areas for the preparations used ranged from 69 to 71 mm²) It is however improbable that the unstirred layer during Ringer perfusion had this thickness because a complete equilibration of so thick a surface layer through pure diffusion in a free water phase is impossible for a molecule as large as dextran ($\bar{M}_W \approx 250\ 000$) It is more likely that in the Ringer perfusion period the unstirred layer was thinner since as is evident from Fig 29 the equilibration volume for sucrose after 100 msec Ringer perfusion is somewhat greater than for dextran Assuming that the diffusion co

efficient is the same as in the free aqueous phase in 100 msec NaCl would attain equilibrium with a layer having a thickness of about 7μ (calculated as diffusion in a plane sheet see CRANK 1956) The amount of dextran which would be found in the corresponding volume (assuming the minimal area of the preparation) is of the same order as twice the error of the anthrone method Sucrose and ficoll (Fig 29 p 66) equilibrate in a larger volume than dextran It therefore seems likely that the *thickness of the unstirred layer during Ringer perfusion is from 0 5* (cf p 63) *to about 7μ* In the influx experiments made according to the DMM it was found that after the first active Ringer perfusion period there was a certain amount of the tracer (Na^{24} K^{42} Br^{82}) already taken up by the preparation [1] *It does not therefore appear probable that the unstirred layer during the Ringer perfusion period is the rate limiting step for the distribution of tracer between active solution and the preparation* The distribution of tracer between the active solution and the tissue preparation is discussed under section C (cf also section F)

The volume of the extra cardiac surface film O_R during extended silicone oil perfusion

As is evident from the decimal pip recording in Fig 23 (p 47) the boundary between active Ringer perfusion and silicone oil perfusion is as far as the radioactive analyses are concerned rather sharp With the change from turbulent Ringer to turbulent silicone oil streams the intervening phase boundary passes the perfusion chamber quickly (cf Fig 13 p 30) As soon as the phase boundary has passed the counts recorded consist of 1) the background activity 2) trapped activity 3) radioactivity in the preparation itself and 4) the radioactivity in the extra cardiac surface film (O_R)

To study the radioactivity recorded in the system immediately after passage of the Ringer silicone oil phase boundary the experiments illustrated in Figs 30 and 31 were performed Sinus venosus from toad was used in one case beating spontaneously and in the other in a non beating condition due to the addition of potassium to

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[1] The experiment illustrated in Fig 24 (p 57) is an exception because a calculation of the counting activity of the surface film O_R plus the trapped activity gave a value of 380 cps A probable reason for this seems that the surface film and/or trapped activity in this experiment were smaller than calculated

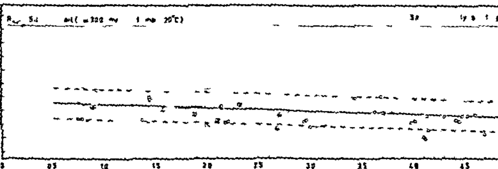


Fig 30 The relative activities measured during the silicone oil periods in an influx experiment (Na) on a spontaneously beating sinus venosus The activities recorded were estimated by the decimal pip system (see text for further details)

the Ringer solution (about 20 mM K⁺) The length of the Ringer perfusion period (t) was 200-300 msec while that of the silicone oil was varied (up to 6 seconds) With the aid of the decimal pip system the mean value for the counting rate during the whole silicone oil perfusion period and the time intervals between each 100 pip impulses was estimated The counting rate for each 100 pip interval was plotted in relation to the mean counting rate for the whole period [1]

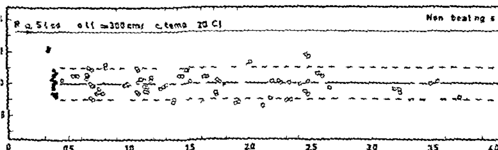


Fig 31 The relative activities measured during the silicone oil periods in an influx experiment (K) on a non beating sinus venosus The solid circles represent analyses not included in the standard error of the estimate (see text for further details)

Because of the limited thickness of the extra cardiac surface film (D_R) the activity of Fig 30 presumably does not truly decrease linearly with perfusion time but it is probable that in a period as short as is illustrated in the figure linearity can be approximately assumed For this reason Bartlett's (1949) method has been used to determine the regression line The standard error of the estimate was calculated as $S = \pm\sqrt{\sum(x-\bar{x})^2/(N-2)}$

In the experiments with a spontaneously beating sinus venosus the relative counting rate decreased by 2% per second for the experiment with Na^{24} (Fig 30) and also for the experiment with K^{42} made on the same preparation. The standard error of the estimate was calculated for Na^{24} as $(S_{Na^{24}})_{method} = \pm 0.11$, for K^{42} as $(S_{K^{42}})_{method} = \pm 0.12$ (according to chapter IV C a 1 a standard error of the estimate $S_{y.x} = \pm 0.10$ could be expected). Fig 31 shows the results for K^{42} in a non beating sinus venosus. The regression line shows that the activity remained constant during the whole of the silicone oil perfusion, the standard error of the estimate being $(S_{K^{42}})_{method} = \pm 0.10$. It was observed that the counting activity during silicone oil perfusion did not always decrease in a spontaneously beating preparation. As was shown in an experiment at +8 C when the radioactivity (Na^{24}) was measured during a silicone oil perfusion period 21 seconds long, the counting rate was maintained during the whole of this perfusion time at the same level (cf also Fig 33 p 72).

It was therefore evident in Figs 30, 31 and 33 that *the counting activity during silicone oil perfusion was relatively constant during each individual experiment with perfusion times of about 1 second* ($t \approx 1$ sec in the influx experiments by DMM).

Summary It is concluded that *during Ringer perfusion the preparation is surrounded by an unstirred layer with a probable thickness of 0.5–7 μ*. During active Ringer perfusion it is improbable that the unstirred layer is the rate limiting step as regards distribution of the tracer between the perfusion fluid and the preparation. *With change over to silicone oil perfusion an extra-cardiac surface film of active Ringer solution (O_k) remains after passage of the Ringer silicone oil phase boundary. This surface film of aqueous solution has the same specific activity as the active Ringer solution at $t = 0$ and has probably a mean size of about 8.5% of the wet weight of the preparation* (the maximal thickness 25–30 μ). A small part of the material of this surface film appears to be transported away during silicone oil perfusion most probably by the turbulent silicone oil stream which may tear off small drops of Ringer solution; the counting activity in the system thus decreases at a rate of between 0 and 2% per second (some distribution of the tracer between the surface film of the preparation and the turbulent silicone oil stream is also possible). *To simplify the discussion the counting activity in the silicone oil perfusion period will*

henceforth be treated as though it were constant because as can be seen in Fig 33 (p 72) the reduction in radioactivity was relatively constant in each separate experiment The sham influx experiments (p 61) indicated that the *trapped activity varied randomly* from perfusion period to perfusion period *Whether the volume of the extra cardiac surface film O_R only exhibits random fluctuations from perfusion cycle to perfusion cycle is difficult to assess* (systematic fluctuations in the volume of this film resulting from the mechanical activity of the preparation cannot be excluded) see also summary section C

C The uptake of tracer in the tissue of the preparation during Ringer and silicone oil perfusion

It was argued in the previous section that there are probably differences in the boundary processes for distribution of the tracer during Ringer and silicone oil perfusion During active Ringer perfusion (I) the tracer can exchange between the solution and preparation with silicone oil perfusion any exchange mainly occurs between the tissue and the extra cardiac surface film (O_R) The exchange kinetics between the perfusing Ringer solution or the surface layer during the silicone oil period and the tissue may be considered as a case of diffusion through a thin film the film thickness however is probably much greater in the latter cases The system used in these flux experiments is basically similar to the material transfer problems encountered in ion exchange or chromatographic processes where there are two main transfer barriers (1) the diffusion within the gel particle (gel diffusion kinetics) and (2) the transfer from outside solution to the gel phase (film diffusion kinetics) *Film diffusion becomes increasingly important as the solute concentration decreases and may become the rate limiting step* These processes are discussed exhaustively by HELFFERICH (1959) (see also TEORELL 1953) The rate of uptake for the isotope studied will be expected to be higher during the active Ringer perfusion period because during this time the preparation is washed continually by a solution with a constant specific activity while during the silicone oil perfusion period the only source of external radioactivity is in the extra cardiac surface film of limited volume and can be expected to diminish somewhat during the silicone oil perfusion a condition which further complicates the issue With more information about the distribution conditions during the differ

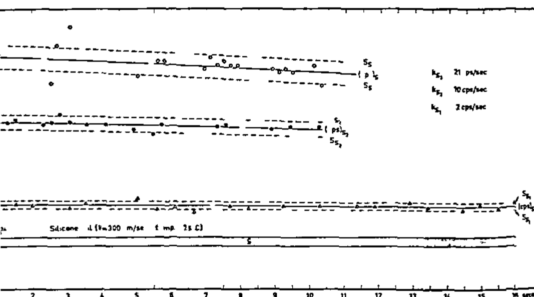


Fig 33 The radioactivity in the system during the long silicone oil perfusion periods S_1 S and S_3 of the experiment of Fig 32 The activity was measured by the decimal pip system (see text) The standard error of the estimate was calculated a

$S_{x1} = \pm 46$ cps (43) $S_{x2} = \pm 86$ cps (84) $S_{x3} = \pm 136$ cps (115)
The figures in parentheses are the S_{stat} values The error in the case of S_3 is large because of the slow film speed used in the recording (higher activity)

ent perfusion phases it might be possible to make a correction in an influx experiment so that the real connection between the increase in activity of the preparation and the active Ringer perfusion could be estimated

An influx experiment (DMM) interrupted with long periods of Ringer or silicone oil perfusion alone

Fig 32 (p II) illustrates an experiment made on a spontaneously beating sinus venosus with active Ringer solution containing Na^{24} ($[R_{Na^{24}}]$ = 159 cps mg) The preparation was perfused alternately with active Ringer solution and silicone oil at a temperature of +7 5°C and a mean perfusion velocity of about 300 cm/sec (t = 100 4 msec t_s = 1320 msec t_m = 1001 m ec) The radioactivities recorded have been plotted as though they occurred instantaneously at the end of the preceding perfusion period The open circles of *curve I* in the figure are the successive means of three consecutive measurements (i e

each point is the mean of the points in the immediately preceding the same and the immediately succeeding measuring periods) This was done in order to smooth out fluctuations The alternate Ringer silicone cycles were interrupted by long Ringer or silicone oil perfusion periods (R_1 R_2 R_3 R_4 and S_1 S_2 S_3 S_4 S_5) Curve I thus describes the influx experiment The increases in activity between b–c d–e f–g and h–i are those which occurred during the long perfusion periods with active Ringer solution (R_1 R_2 R_3 and R_4) and have been interpolated as linear functions of time

Fig 33 illustrates the activity in the long silicone oil periods (S_1 S_2 S_3) in the same experiment (the counting rate was calculated from the time required for a collection of 500 impulses in the decimal pip system cf Figs 30 and 31 p 69) During the silicone period the activity decreases a little with time (see discussion in section B) The decrease occurred more rapidly during S_3 than S_2 or S_1 with values of 0 8 % 0 5 % and 0 2 % per second respectively of the mean activity during the period This can probably be explained by the specific activity in O_R decreasing more slowly during S_3 than during S_2 or S_1

Calculation of the influx resulting from continual active Ringer perfusion

The time scale of *curve I* in Fig 32 (p II) is that of the actual experiment *Curve II* has been plotted by including only the time periods of the Ringer perfusion (t = 100 4 msec) and neglecting the times of the silicone periods The solid circles are the individual measurements made during the alternating silicone oil periods and lie in the intervals *ab cd ef* and *gh* Each interval represents the total time for which the preparation was in the perfusing Ringer solution during that part of the experiment The increases during these periods are faster than in the long pure Ringer periods (*bc de fg hi*) which is interpreted as indicating that the time scale in these sections (*ab cd ef gh*) is false In these parts of the actual experiment the distribution of tracer to the tissue has also occurred during the silicone oil period from the extra cardiac surface layer O_R Assuming that the intervals *bc de fg* and *hi* represent the real conditions with pure long active Ringer perfusion a true scale can then be calculated for *ab cd*, *ef* and *gh* The result of this is shown in *curve III* The rate of uptake in the section *bc* of *curve II* is $(\Delta \text{ cps})_{bc}/t_R$ where $(\Delta \text{ cps})_{bc}$ is the in

crease of activity during t_R where t_R = actual time of active Ringer perfusion. Hence for *de* we have $(\Delta \text{ cps})_{de}/t_R$ etc. During the interval *cd* on the other hand the actual distribution time for the tracer between the tissue and the active Ringer phase (active Ringer perfusion during t_r and the surface film O_R during t_s in each cycle) is equal to $\Sigma t_r + \Sigma t_s$; in curve II only Σt_r was used. For the construction of section *cd* of *curve III* the following expression was used

$$\frac{(\Delta \text{ cps})_{cd}}{\sum t_r + \sum t_{r_{eqv}}} = \frac{\frac{(\Delta \text{ cps})_{bc}}{t_R} + \frac{(\Delta \text{ cps})_{de}}{t_R}}{2} \qquad (1)$$

In this way one can calculate the additional time required (Σt_{eqv}) to adjust the distribution rate under this section of the curve (II) to the "true" value providing there were a constant specific activity around the preparation during this time. Curve III (Fig. 32) has been plotted by adjusting the relevant sections of curve II in this way (*ab*, *cd*, *ef* and *gh*).

From the uptake rates of periods *ab*, *cd*, *ef* and *gh* in curve I a new curve (IV) has been drawn in which corrections have been made for the regions of the long Ringer (*P*) and silicone (*S*) where the alternate Ringer–silicone period pattern was interrupted. *Curve IV* thus represents the result that would have been obtained if the alternate perfusion periods t_r and t_s had not been interrupted.

Fig. 34 (p. II) is a schematic representation of Fig. 32 and illustrates graphically the arguments presented above. Curve IV, as in Fig. 32, represents the theoretical increase in activity as a function of time if the alternate Ringer–silicone oil perfusion had been maintained during the whole experiment (t_r/t_s = const.). Curve II is drawn schematically while curve III is that calculated as in Fig. 32 for the approximately "true" relationship between the increase in activity of the preparation and time required if there had been continual perfusion with active Ringer solution (t_R). Sections *ab*, *cd*, *ef* and *gh* were calculated in this way. For example in section *cd* the activity increase during alternate perfusion ($\Sigma t_r + \Sigma t_s$) was 450 cps with

$$\frac{t_r}{t_s} = \frac{\sum t_r}{\sum t_s} = \frac{100.4}{1320} = 0.076$$

Now an increase by 450 cps would also occur with a perfusion period of Ringer alone and the length of this period would be t_R and t_R =

$\Sigma t + \Sigma t_{r_{eqv}}$ (equation 1) During Σt_r the increase can be calculated as 80 cps (curve III) The remaining activity of 370 cps arose therefore in Σt_s by distribution of the tracer between the preparation and the surface film (O_R) during silicone oil perfusion *As can be seen in the schematic construction the ratio* (Δ cps)$_{\Sigma t}$ /(Δ cps)$_{\Sigma t}$ $\approx$ *constant with the assumption that* t/t_s *is also constant during the whole experiment This is a necessary requirement for a c-a t* (see chapter VI d)

The experiment illustrated in Figs 32 and 34 is the only one of this type made but the results are so clear that they should aid in the understanding of the problems involved

The mean concentration of Na^{24} *in the extra cardiac surface film* (O_R) *during silicone oil perfusion*

The data of the experiment of Figs 32 and 34 (p II) can be used to calculate the mean concentration (= cps Na^{24} per mg of surface layer) of Na^{24} in O_R provided certain assumptions are made The agitation which almost certainly occurs in this layer due to the adjacent turbulent silicone oil stream (cf pp 63 68–71) may tend to create homogeneity in the Na^{24} concentration

Assuming that the volume of O_R is constant (cf summary of section B) and that at $t_s = 0$ the specific activity in O_R is equal to that of the active Ringer solution (cf p 70) the following equation is valid (conservation of tracer)

$$(C_o)_1 \cdot V_o - (C_o)_2 \cdot V_o = (C_p)_2 \cdot V_p - (C_p)_1 \cdot V_p \qquad (2)$$

where

V_o = volume of O_R (assumed to be 8 5% of the wet weight)
V_p = volume of the heart preparation (density = 1)
$(C_o)_1$ = tracer concentration (cps/mg) in O_R at time $t_s = 0$
(C_o) = tracer concentration (cps/mg) in O_R at time t
$(C_p)_1$ = tracer concentration (cps/mg) in the tissue at $t_s = 0$
(C_p) = tracer concentration (cps/mg) in the tissue at time t

and

$$\underbrace{V_o \; [(C_o)_1 - (C_o)_2]}_{\text{amount of tracer leaving } O_R \text{ during } t \text{ msec of silicone oil perfusion}} = \underbrace{V_p \; [(C_p)_2 - (C_p)_1]}_{\text{amount of tracer entering the preparation during } t_s \text{ msec of silicone oil perfusion}} = \Delta \text{cps} \qquad (3)$$

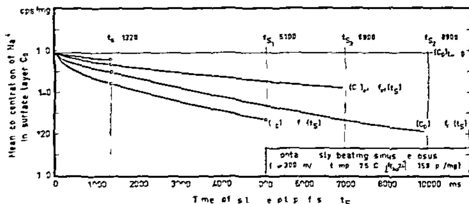


Fig 35 Calculated mean concentration (C_o) of Na^{24} in the external surface film (O_g) surrounding the tissue preparation during silicone oil perfusion The topmost curve corresponds to $(C_o)_{gh} = f_{gh}(t)$ The point for $(C_o)_{ef}$ was not used since a large error might have been introduced because of the very small slope of the curve in this range For further details see text

Equation (3) is valid if both V_o and V_p are constant and can be re written

$$(C_o)_2 = (C_o)_1 - \frac{\Delta\,cps_t}{V_o} \tag{4}$$

In this equation $(C_o)_1$ is the concentration of the active Ringer solu tion (cps/mg) $V_o = 8.5\,\%$ of the wet weight of the preparation (mg) and Δ cps is its activity increase during silicone oil perfusion (cps) By determining (C_o) in the interval *ab* *cd* *ef* and *gh* and utilizing the single measurements made after the long silicone oil perfusion periods (S_1 S and S_2) in the experiment of Fig 32 (p II) the mean concen tration of Na^{24} in O_R was calculated the results are given in Fig 35

In the experiment (Fig 32) $(C_o)_1 = 159$ cps/mg $V_o = 1\,99\,\mu l$ (= mg) Δ cps can be obtained from the calculated increase in activity of the preparation during the intervals *ab* *cd* *ef* *gh* as illustrated in Fig 34 (p II) $(\Delta\,cps)_{cd}$ for $t = 1320$ msec was calculated from the increa e in activity during silicone oil perfu ion [$(\Delta\,cps)_{\Sigma t} = 370$ cps] and the number of silicone oil periods ($N_t = 19\,7$ during *cd* curve I) [1] The value of $(C_o)_2$ is then calculated as for example in the interval *cd* for $t = 1320$ (cf eqn 4)

[1] The number of silicone oil perfusion periods 1320 msec long in the different intervals of curve I of Fig 34 were *ab* 21 *cd* 20 *ef* 38 and *gh* 46 The calculated increase in activity of the preparation during silicone oil perfusion for the cor responding intervals is shown in Fig 34 $(\Delta\,cps)_{\Sigma t}$

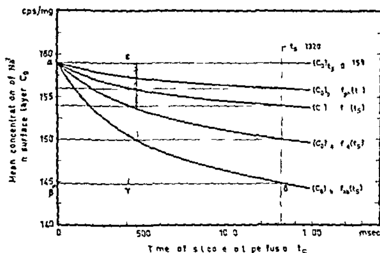


FIG 36 Graphical construction of the mean concentrations (C_o) in the external surface film (O_R) during silicone perfusion between $t_s = 0$ msec and $t = 1320$ msec for different periods of the influx experiment illustrated in Figs 32 and 34 (p II) The t_{req} values ($\gamma - \beta$ in the figure) were for the intervals ab 411 msec cd 469 msec ef 463 msec and gh 465 msec The mean value for t_{req} was 450 msec

$$(C_o)_{t\ 1320} = 159 - \frac{(\Delta\,\text{cps})_{ut}}{\Delta t\ \ I} - 159 - \frac{370}{19\,7\ \ 1\,99} = 150$$

By using the measurements made immediately after the end of each long silicone oil perfusion period [S_1 S_2 and S_3 in Fig 32 this is the radioactivity measured during the first ordinary silicone period (1320 msec) after the first active Ringer period (100 4 msec) after S_1 S or S_3] estimates of Δ cps, were obtained The values calculated are drawn in Fig 35

Fig 36 is a graphical construction of the mean Na⁺ concentration in the layer O_R during silicone perfusion (I =constant) The ordinate also represents the amount of tracer which leaves O_R and equation (4) can be written

$$\Delta\,\text{cps}_s\quad I\quad [(C)_1 - (C_o)_2] \qquad (4\,a)$$

It is assumed that for only a very short time after $t = 0$ the distribution in the tissue is at the same rate as with the active Ringer perfusion (t) and that the mean concentration of tracer in O_R at t_s = 1320 msec can be obtained from the functions shown in Fig 35 The

quantity of tracer which during 1320 msec of silicone oil perfusion (δ–β in the figure) entered the preparation in different periods of the influx experiment (*ab cd ef gh*) can be expressed as the quantity of tracer which would enter during $t_{r_{eqv}}$ (in active Ringer) for the corresponding period of the experiment (γ–β) (cf Fig 34)

The uptake of Na⁺ in the tissue during active Ringer and silicone oil perfusion

Fig 37 shows the calculated increase in activity of the tissue during Ringer and silicone oil periods at different intervals during the experiment (see Figs 34 and 36) For section *ab* (Fig 34) $t_r = 100\,4$ msec (α in Fig 37) the increase is 6 4 cps and for $t_s = 1320$ msec (γ in Fig 37) the corresponding value during silicone oil perfusion 27 4 cps The intercept β gives $t_{r_{eq}}$ during interval *ab*

Fig 37 shows that the activity increases during Ringer and silicone oil perfusion follow different functions with respect to time If these results are generally valid *the figure should serve as a nomogram for calculating the true time relations between increase of activity in the tissue and the active Ringer perfusion time for other influx experiments* where t/t_s has other values and where ions with approximately similar properties to Na have been used (cf p 88)

Reconstruction of an interval of an influx experiment

With regard to the arguments developed (Figs 32–37) a *reconstruction of a small part of interval ab of Fig 32 (p II) will now be described* Fig 38 illustrates three Ringer silicone oil perfusion cycles Curves II III and IV have been taken as linear over this short time period The lower part of the left ordinate shows the total Na^{24} activity in the surface film (O_R) and the amount of trapped activity which has been assumed constant (cf Fig 27 p 62) V_e and $(C)_{t=0}$ have also been taken as constant i e $V \cdot (C)_{t=0} = 1\,99 \times 159 = 316$ cps (background activity also assumed constant) The right ordinate gives the Na^+ activity measured during silicone oil perfusion [zero = counting rate–background (186 cps)] Cps_{s1} cps_{s2} and cps_{s3} are the activities recorded during the silicone periods *s*1 *s*2 and *s*3 which followed the Ringer perfusion periods *r*1 *r*2 and *r*3 It is further assumed that the trapped volume and the surface film (O_R) had attained the specific activity of the active Ringer solution instantaneously at the first active Ringer perfusion period (*r*1) The different time scales have been

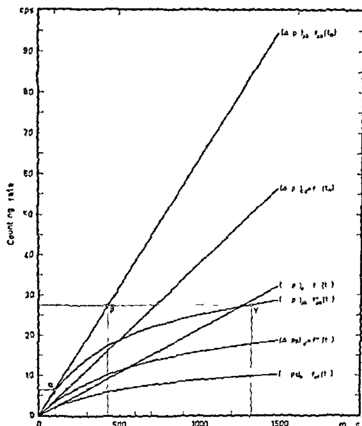


Fig 37 The increase in activity in the tissue of the preparation during different stages of the influx experiment of Fig 32 (p II) Values for both Ringer and silicone oil perfusion are given The increase of activity during active Ringer perfusion does not follow the same time course as during silicone perfusion The figure can be used as a *nomogram* for the calculation of t_{eq} in experiments where t and t_s have different values t_q is the intercept (β) of the estimated *counting rate* (γ) at t *with the increase in counting rate with active Ringer solution alone*

illustrated schematically as in Fig 34 (p II) i e for curve IV during Ringer and silicone oil phases during alternate perfusion (top time diagram under abscissa) and for curve III under hypothetical continuous Ringer perfusion ($t + t_{t_{eq}}$) The upper part of the left ordinate represents the Na^{+} activity in the tissue The increases of activity are assumed to be identical in each of three Ringer silicone oil cycles the increase during the Ringer period (t_r = 100 4 msec) is 6 4 cps and in the silicone period (t_s = 1320 msec) 27 4 cps as calculated in Fig 34

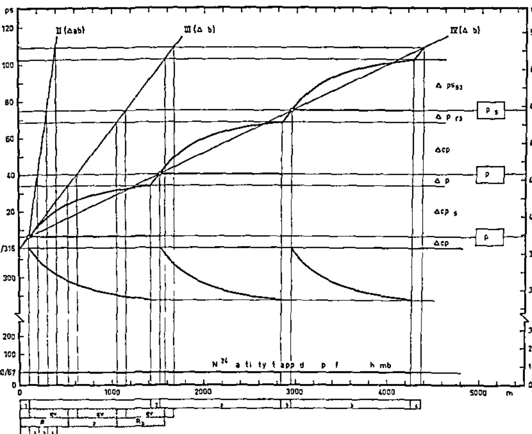


Fig 38 Reconstruction of a small part of the interval *ab* of Fig 3 (p II) The right ordinate gives the total activity recorded after subtraction of the background (background = 186 cps corresponding to 1 17 mg active Ringer solution) *Note* Both the left and right ordinate scales are interrupted For further details see text

(p II) and Fig 37 (p 79) The activity recorded during each silicone period is arbitrarily always placed at the beginning of the period ($t_s = 0$ open circles) the point of time at which the activity becomes locked in by the silicone (passage of Ringer–silicone phase boundary) There is assumed to be no loss of activity from the chamber during the silicone oil perfusion period

If V_o *and the trapped volume are constant (or vary purely randomly from cycle to cycle) the activity in the tissue can be determined with the greatest certainty at* $t = 0$ when under the above conditions it is correct to write

$$cps_{ti} \quad cps - (cps_{O_R} + cps_{\text{trapped activity}} + cps_{\text{background}})$$

where cps_s = total activity recorded during a silicone oil period and cps_{O_R} = total activity of surface film O_R at $t = 0$ and

$$(cps_{O_R} - cps_{\text{unspec. activity}} - cps_{\text{background}}) = \text{constant}$$

During silicone oil perfusion there will be some redistribution of radioactivity between the surface film (O_R) and the tissue. The tissue increase (Δcps in Fig. 38) cannot be determined before the next analysis period during the succeeding silicone period. The same amount of tracer (Δcps) must also leave O_R. During the active Ringer perfusion there will be a net tracer movement (Δcps) into the tissue compartment and after each such period the total activity in the surface layer (O_R at $t = 0$) will be 316 cps.

The tissue activity (cps_{tissue}) *during the silicone–Ringer cycles can be described in the average case by the function represented by the thick line between the open circles in the figure. The difference in activity recorded between two analysis points depends on the particular type of distribution function operating during the intervening period.* This also imposes limits on the time intervals during an influx experiment for which measured activity differences are representative.

In the influx experiments the entire activity measured during a silicone oil perfusion period is assigned to the end of the previous Ringer period (r) *the maximal conceivable shift of the point would be by a time* ($t + t$) *to the left i.e. one whole silicone Ringer cycle earlier.*

With the use of a c-a t it is implicit in applying the interpolation procedure that the distribution functions in all the individual silicone–Ringer cycles are the same (chapter VI e).[1]

The assumptions regarding the constancy of the volume of the surface layer O_R requires further comment. *Since the activity during silicone oil perfusion is virtually constant* (see summary of section B) *the volume* (V) *of O_R will be determined when the Ringer–silicone phase boundary passes the tissue preparation. If the volume of O_R is correlated to the phase of the mechanical activity the latter must also be correlated*

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[1] The whole of the increase of activity in the preparation (≈ 34 cps) measured as the difference between two successive measurements occurs before the time to which the increase is attributed. About °3 of the influx occurs linearly during t [t = 0 0˜6 (t t)] and the remaining ˜˜ exponentially from O_R during the silicone oil phase (t). Thus the 50 influx point occurs earlier than the mid point of the silicone Ringer cycle preceding the time point to which the subsequent *measurement is assigned*

with the passage of the interface (i e the time point to which radioactive measurements have been assigned in all influx experiments)

The DC and AC components during an influx experiment

In an influx experiment with alternate Ringer–silicone oil perfusion (DMM) of a sinus venosus preparation mounted in a chamber the radioactivity recorded increases with respect to both the times of perfusion of the Ringer and silicone oil If all fluctuations are neglected an exponential mean curve is obtained (DC component , see chapter VI e) The influx kinetics during the individual Ringer and silicone oil periods are different this presumably depends on different boundary conditions During the Ringer period the preparation is surrounded by a solution of constant specific activity (infinitely large source) In the silicone oil phase the tracer can only distribute between the tissue and a surface layer of limited volume whose specific activity must decrease (providing the tracer diffuses down its concentration gradient)

The time course of the DC component depends on 1) the electro mechanical state of the tissue and 2) the perfusion programme i e the relationship between the lengths of the Ringer (t) and silicone oil (t) periods i e t_r/t_s As can be seen in Fig 24 (p 52) the DC curve was steeper in a spontaneously beating than in an electro mechanically inactive preparation Assuming t/t_s remains constant during the whole experiment the DC component with a non beating preparation should represent the result of a passive redistribution of tracer in the tissue under the perfu ion conditions operating (passive distribution base line) With increase of t/t_s the slope of this passive DC line should be steeper the steepest curve obtained was that during continuous Ringer perfusion (cf curve III in Fig 32 p II and curve II in Fig 25 p I)

Superimposed on the DC line is an AC component (see chapter VI e) *Fluctuations around the mean trend line (= base line) consist to a great extent of random noise* (see chapter VI e) i e the resultant of variations in the analysis system the perfusion system (trapped activity) background activity random variations in the value of the extra cardiac surface layer (O_R) and the statistical counting error *It was calculated with the e-a t method that the AC component also exhibited systematic fluctuations of radioactivity correlated with the electro mechanical activity cycles of the spontaneously beating preparation*

Systematic fluctuations in the activity of the spontaneously beating preparation might arise from events not directly connected with transmembrane ion movements, i.e. 1) there may be a correlation between the volume of the external film (O_R) during silicone perfusion and the mechanical activity of the preparation and 2) *the distribution and movements of tracer with the tissue may depend on both mechanical* (e.g. geometrical changes of extracellular matrix) *and electrical events* (e.g. causing alterations in the physico-chemical properties of the extracellular space).

The time relations between the measured radioactivity pattern and the electro-mechanical activity for a mean cycle

The radioactivity measured during the silicone period (DMM) is arbitrarily assigned to a time point at the very beginning of that silicone oil period, i.e. at the termination of the period whose influx is being determined. The start of this period was $(t - t)$ seconds earlier.

If the tissue activity pattern depends on some correlation between O_R and the phase of mechanical activity a time correction for the cycles of the latter is not necessary because the radioactivity measurements are made during a period when the total activity does not change (t_s) and assigned to the time at which the Ringer–silicone interface passes (cf. p. 81).

If the fluctuations estimated by the c-a t are due solely to variations in the volume of O_R then under all circumstances the patterns for all isotopes studied by the DMM will have the same general relationship to the electro-mechanical events independently of the relationship between t and t_s.

If the distribution of the tracer in the tissue is affected by the electro-mechanical activity then different isotopes would quite likely not behave in the same way and the activity pattern would probably be correlated with the t/t_s ratio. In this case a correction for the time relation between the mean activity cycle and the electro-mechanical events might be necessary (though difficult to calculate). *It seems probable that the mean cycles for Br^{82} and Na^{24} (Figs. 26 p. 56, 47 p. 117 and 48 p. 118) should be placed a little earlier in relation to the electro-mechanical cycle* (cf. general discussion p. 120).

Summary. The time course of the radioactivity recorded in an influx experiment (DMM) on a spontaneously beating sinus venosus

has two components a mean exponential (DC) curve on which are superimposed fluctuations (AC component) The form of the DC component depends on the electro mechanical activity of the preparation and the perfusion conditions (t_r/t_s) The AC component consists both of random fluctuation noise and systematic fluctuations correlated with the electro mechanical activity of the spontaneously beating tissue Systematic fluctuations in the radioactivity around the base line might arise from processes not directly connected with transmembrane ion movements There may be a correlation between the volume of O_R and the mechanical activity of the preparation or some relationship between the electro mechanical activity and the distribution of tracer in the preparation If the activity pattern calculated by the *c a t* method resulted solely from variations in O_R the activity patterns for the different isotopes studied with the DMM should have essentially the same form and relation to the electro mechanical events If there is a correlation between the electro mechanical activity and the distribution of tracer in the tissue it is likely that with different isotopes both the pattern of radioactivity and its relationship to the electro mechanical events would differ Further in the latter case the relationship between the electro mechanical events and the radioactive pattern would probably depend on the perfusion conditions (the value of the t_r/t_s ratio)

D The equilibration volumes and uptake rates during an influx experiment

A review and analysis of ion exchange kinetics between two different phases in a multi compartment system has been recently published by HAAS (1962) By way of introduction to the experiments which were carried out with the perfusion and soaking methods described in chapter V a short review of certain relevant literature concerning the distribution of electrolytes and non electrolytes between bathing solutions and tissue preparations will be given Because of the type of preparation and perfusion techniques used the results vary within rather wide limits

CAREY and CONWAY (1954) showed that a rapid sodium efflux from the frog sartorius probably came from the extracellular space SCHREIBER (1956) described both influx and efflux experiments on frog ventricle and concluded that the K^+ efflux had two components

a rapidly escaping fraction with $T\frac{1}{2} = 22$ min and a slower fraction with $T\frac{1}{2} = 80$ min Similarly for the influx there were al o two velo cities involved a rapid component with $T\frac{1}{2} = 15$ min and a slower one with $T\frac{1}{2} = 68$ min The Na^{24} efflux was al o dual in nature with a rapid component (comprising 91 % of the total Na^{24}) with $T\frac{1}{2} = 4$–4.5 min (interpreted as representing the extracellular Na^{24}) and a slower fraction with $T\frac{1}{2} = 170$ min JOHNSON (1957) found that the Na in flux into the frog ventricle reached a plateau within 10 minutes HARRIS (1957) claimed that equilibration of the extracellular space in the toe muscle (weight about 3 mg) from the frog took place within 1 min HUTTER (1961) found that the efflux of K^{42} from the extracellular space in the sinus venosus of the frog was virtually complete within three minutes with perfusion of frog ventricle CRAY LAMB and McCLIGAN (1964) found rapid clearing of the extracellular space HAAS and GLITSCH (1962) studied the potassium flux in frog auricle (thickness about 0 3 mm) and both the influx and efflux were of a double exponential type the $T\frac{1}{2}$ value for inulin efflux was 1 7 min HAAS GLITSCH and TRAUTWEIN (1963) studied Na^{24} efflux from frog auricle and claimed that there were three components the first rapid fraction was interpreted as being due to expulsion from the extracellular space (it was dependent on electro mechanical ac tivity) LANGER and BRADY (1963) found that in the mammalian ventricle the influx of calcium reached a steady value within 20–25 min NIEDERGERKE (1963) studied Ca^{45} influx into frog ventricle in a normal sodium environment Ca^{45} reached a steady level within 5 minutes while the sulphate inulin and sodium spaces were about the same size the calcium space wa larger The Ca^{45} efflux was cor related with the stimulation frequency

Equilibrial volumes and times for soaking experiments

Fig 39 illustrates an experiment where a spontaneously beating atrium and sinus venosus were bathed for different periods in active Ringer solution which contained Na^{24} Br^{82} and raffinose (solution III c) The solution was vibration stirred and the method was as described in chapter V A b The experiment was one of two which were made both yielding the same results The equilibration volumes were calculated assuming that they contained the same concentra tion of test solute as in the active Ringer solution [1] and the data are

[1] No correction was made for the Donnan distribution of electrolytes be

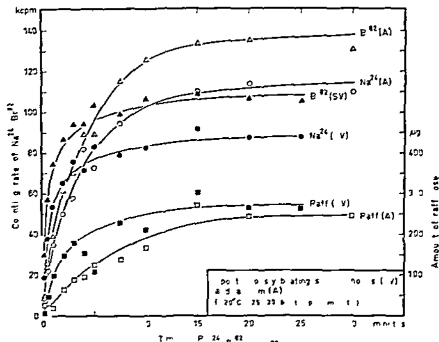


FIG 3? Influx experiment for Na^{24} Br^{82} and raffinose on a spontaneously beating sinus venosus (SV) and atrium (A) by the procedure described in chapter V A b Wet weights sinus venosus 14 0 mg atrium 20 8 mg Analysis accuracy for Na^{24} and Br^{82} better than ±0 5 Activities of solutions [Na^{24}] = 7033 cpm/mg [Br^{82}] = 7021 cpm/mg the raffinose concentration was 20 μg/mg (FSA values 5? kcpm Na^{24}/μmole Na 581 kcpm Br^{82}/μmol Br) External surface layer (O_P) calculated as 8 % of the wet weight $(O_R)_{SV}$ = 1 19 mg equivalent to 8 4 kcpm Na^{24} 8 4 kcpm Br^{82} and 24μg raffinose $(O_R)_A$ 1 77 mg equivalent to 12 6 kcpm Na^{24} 14 0 kcpm Br^{82} and 3 μg raffinose The equilibration levels in the figure are Sinus venosus (SV) Na^{24} 82 kcpm Br^{82} 102 kcpm and raffinose 260 μg Atrium (A) Na^{24} 107 0 kcpm Br^{82} 130 0 kcpm and raffinose 23 μg (1 kcpm = 10 cpm)

given in the figure text [1] It can be seen in the figure that Na^{24} Br^{82} and raffinose equilibrated relatively rapidly with a space which was considerably greater than that of the extra cardiac surface film (cf Fig 28 p 65) In general it can be said that the equilibration level was reached more rapidly for sinus venosus than for atrium The

tween Ringer solution and the extracellular space of the preparation the theoretical Donnan factor for human plasma and protein free ultrafiltrate has been calculated and found to be approximately 0 96 for univalent ions (for reference see FRIEDSTRÖM 1962) Cf also DANIELSON (1964)

[1] The backward projection technique for graphical resolution of exponential processes in the determination of the rate constants was not used here because of the rather coarse technique used see LIEW (1962)

longer equilibration times for atrium depend probably on the larger equilibration volume It is also clear that the distribution of raffinose in both cases was slower than that of Na^{+} and Br^{-} This is not unexpected in view of the different diffusion coefficients for the substances[1]

The raffinose Ringer solution was hypertonic (raffinose concentration = 34 mM) BOZLER (1961 *b*) with frog sartorius showed that a sucrose Ringer solution (80 mM sucrose) produced a reduction in weight of about 14 % after a soaking time of 2 hours It was shown also that sucrose entered into the extracellular space and probably also intracellularly (BOZLER 1959 1961 *a*) The weight loss recorded therefore is presumably the resultant of a rather complex system of forces The osmotic effect of the raffinose Ringer solution (34 mM raffinose) was therefore studied in a soaking experiment with the determinations of the changes in wet weight after different lengths of soaking time Inverted spontaneously beating sinus venosus and atrium were mounted in the same way as shown in Fig 22 a toe muscle from the toad was also used After soaking for 30 minutes at room temperature the wet weight reductions were for sinus venosus 7 % atrium 5 % and toe muscle 5 % (of the original weight in normal Ringer solution—solution I) after 2 hours soaking the values were 15 % 8 % and 10 % respectively

The equilibration levels shown in Fig 39 (see figure text) include an extra cardiac surface layer O_R which was calculated as equivalent to 1 19 mg of solution for the sinus venosus preparation and 1 77 mg for the atrium (8 5 of the wet weight) After subtraction of the quantity of activity in O_R the equilibration volumes for the preparations were calculated (cf footnote p 85) as sinus venosus raffinose = 11 8 mg Br^{82} = 11 8 mg atrium raffinose = 10 0 mg Br^{82} = 14 6 mg

DANIELSON (1964) measured the bromide space after 30 hours equilibration *in vivo* (*Bufo bufo*) and using his values (calculated without a correction for the Donnan distribution) the Br^{-} space in the sinus venosus used above should correspond to 9 3 mg ± 1 1 mg and in the atrium 11 3 mg ± 1 0 mg (the Br^{-} and I^{-} spaces were essentially the same as that of Cl^{-} in sinus venosus auricle ventricle

[1] D_{NaCl} (concentration = 100 mM) = 1 48 × 10^{-5} cm^2 sec^{-1} and D_{NaBr} (100 mM) = 1 51 × 10^{-5} cm^2 sec^{-1} at 25 C (ROBINSON and STOKES 1955) and $D_{raffinose}$ = 0 4 10^{-5} cm^2 sec^{-1} at ± 18 C (CONWAY 1959)

and skeletal muscle) Assuming that Br^{8} is mainly extracellular the equilibration volumes for Br^{8} and raffinose for the sinus venosus are in good agreement though perhaps somewhat greater than DANIELSON's values As regards the atrium there is good agreement between the equilibration volume for raffinose and the extracellular space but the equilibration volume for Br^{8} is higher than given by DANIELSON A calculation of the equilibration volume for Na^{24} in the experiment of Fig 39 gave for the sinus venosus 10 4 mg and for atrium 13 3 mg the values calculated from DANIELSON's (1964) data were 8 8 $\pm$ 1 3 and 11 2 $\pm$ 0 9 respectively Although the technique used was rather coarse the results are in general in good agreement with those reported in the literature though two potential sources of error must be noted The volume of O_R was not determined for each individual preparation and further in determining the wet weight any Ringer solution which was trapped between the epicardial surface of the preparation and the ECG catheter was not taken into account (cf Fig 15 p 34)

To summarize it may be said that the equilibration volumes for Na^{24} Br^{8} and raffinose in sinus venosus were in good agreement with the extracellular anion space estimated by DANIELSON In the atrium the agreement was good for raffinose but there were small differences for Br^{8} and Na^{24} The rate of equilibration of the extracellular space in sinus venosus was in good agreement with the value given by Hutter (1961) To what extent the intracellular space is involved in the experiment of Fig 39 is difficult to assess For sinus venosus and atrium the intracellular quantities of Na and Br are according to DANIELSON (1964) very small and with the method used for the experiments of Fig 39 would therefore not be measurable

Equilibrial volumes and times for perfusion experiments (DMM)

The volumes and rates of equilibration obtained with the perfusion technique at the temperature which was used for the influx experiments (DMM) require some comment In for example a Na^{24} (Fig 32 p II) influx experiment the true influx curve during continual active Ringer perfusion was calculated (cf Fig 32 curve III)

For the Br^{8} *influx experiment* (Fig 25 p I) $t_{r_{eqv}}$ was calculated from Fig 37 (p 79) with the assumption that the $D_{N\,Br} \approx D_{N\,Cl}$ (cf footnote on p 87) and the correction of the time scale

was made by using the value of t_{eqv} so obtained (time scale for curve II in Fig 25 can be derived from the equation given in the figure) The equation for describing curve II in Fig 25 was obtained by the same procedure as used for determining that of the mean trend line curve I in the same figure (see chapter VI e) the equilibration level corresponded to 7850 cps obtained at $(t + t_{eq}) \approx 200$–250 seconds (+5 C) the equilibration volume after subtraction of the activities of the trapped volume and the extra cardiac surface film (O_R) was calculated as 12 3 mg The value calculated from DANIELSON s (1964) data was 10 7 ± 1 3 mg

As is evident from Fig 32 curve III the Na[1] *influx experiment* did not reach an equilibrial value In order to assess the magnitude of the equilibrial level the equation for curve III describing the relation between increase in activity in the preparation and perfusion time in active Ringer solution was calculated in a similar way as curve II of the Br^{82} experiment of Fig 25 (p I) The equation could then be used to calculate the *approximate* values for the equilibrial level and time the equilibrial level corresponded to 3550 cps and was reached at $(t \ \ t_{eq}) \approx 200$–$250$ sec (+7 5°C) After subtraction for the activity of the trapped volume and O_R it was calculated as 10 8 mg The value calculated from DANIELSON s (1964) data was 14 8 ± 2 2 mg

The calculated equilibrial volumes for Br^{8} and Na^{24} (DMM) thus are in essential agreement with the results of the soaking experiments (Fig 39 p 66) The somewhat higher values for the equilibrial volumes for Br^{8} and Na^{24} can perhaps be explained by the uncertainty in the calculated volume of the extra cardiac surface film and the fact that with determination of the wet weight of the preparation any volume of Ringer solution trapped between the epicardial surface of the preparation and the ECG catheter was neglected (cf Fig 15 p 34)

In the influx experiment made with solution I or II (DMM) no great variation between either the volumes of the intra and extra cellular spaces or the ion concentrations should be expected JOHNSON (1957) showed that the sucrose space was constant for about 2 hours when a frog ventricle was perfused with normal Ringer solution intracellular Na concentration was also apparently unaltered[1]

[1] CAREY and CONWAY (1954) showed however that the Na and Cl content in isolated sartorius muscles increased with time during equilibration in Ringer

Summary In the influx experiments made by the DMM it is probable that with a perfusion temperature of between +5°C and +7 5°C the uptake of tracer is primarily in the extracellular space (equilibrial time for sinus venosus about 200–250 sec during continual Ringer perfusion) It is probable that the uptake rate during perfusion (DMM) was somewhat higher than is the case with more conventional techniques (soaking experiments see Fig 30 p 86) where equilibration of the extracellular space in sinus venosus at about +20 C took place within 3–6 min for Na^{24} and Br^{82} It appears therefore that a somewhat more rapid uptake of the tracer occurs with the perfusion method (DMM) than with conventional soaking

E Studies of the distribution of K^+ ions between the perfusion solution and the outermost cells of the tissue preparation

A method has been previously described for the determination of the size order of the time lag which can be expected with influx measurements (DANIELSON ÖBRINK and SJOSTRAND 1962)

A high outer concentration of K^+ is known to depolarize excitable membranes (for references cf HIGMAN PODLESKI and BARTELS 1963) including heart tissue (WEIDMANN 1956 *a* *b*) With a K^+ rich isotonic Ringer solution KOTOWSKI ANTONI WAHLENKAMP and FLECKENSTEIN (1961) found that the minimal K^+ concentration for depolarization of the frog sinus venosus was about 22 mM K^+

The time interval between contact of the preparation with a Ringer solution containing 44 mM K^+ (solution IV) and depolarization (estimated from the ECG) should give some information about the proximity of the outermost cells to the perfusion solution (cf chapter II pp 13 15) The main part of this time difference can probably be attributed to the time elapsing between contact of the preparation with the potassium Ringer solution and the depolarization of the outermost cells which thus acted as a trigger focus for the rest of the tissue The outermost cells are however those of greatest interest since their superficial position presumably means that they will be very much involved in any intracellular influx which occurs

The sinus venosus (or atrium) was mounted as described in chapter

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solution HAAS GLITSCH and TRAUTWEIN (1963) in a perfused toad atrium found that during the first two hours the total Na increased while the K decreased

TABLE 4 K+-depolarization time lag at 5 C

Exper.ª No.	Preparation	K-time lag in msec	Number of determination	Range
640319 I	sinus venosus	0	6	30–330
640311 II	sinus venosus	1 0	C	130–[illegible]0
640319 V	sinus venosus	140	11	8[illegible]–180
640319 III	atrium	370	3	340–480

ª Each experiment represents measurements on one preparation

II b and perfused in the same way as with the DMM (see chapter V A a) The preparation was perfused with inactive Ringer solution and then with silicone oil (the ECG was recorded continuously) The silicone oil perfusion was interrupted by a perfusion period with K+ Ringer (solution IV 44 mM K) The time of first contact of the K+ Ringer solution with the tissues of the preparation could be determined with the technique described in chapter IV D a 2 The mean lag time found in this way was for sinus venosus perfused at +5 C ≈ 200 msec (see Table 4)

The time lag measured as described above presumably corresponded to the time required for the potassium ions to reach the cellular membranes together with the time required for their depolarization (depolarization should occur when the extracellular K+ concentration is about 22 mM)[1] Even if the depolarization time of the membrane varies depending on the phase of the cardiac cycle when the high potassium concentration reaches the cell membrane the distribution time between the perfusion solution and the cellular membrane will be the same in all cases Thus the real time lag will never exceed the observed values DANIELSON ÖBRINK and SJÖSTRAND (1962) found that the time lag for sinus venosus was 100 msec and for atrium 400 msec using a perfusion technique most nearly resembling that used

[1] In studies of the effect of potassium on the depolarization of the electroplax of *Electrophorus electricus* HIGMAN PODLESKI and BARTELS (1963) found that the membrane potential appeared to be most sensitive to changes in potassium concentration

in the experiment where the preparation was bathed in a soaking solution (cf Fig 39 p 86) The potassium concentration used in this experiment was 125 mM K^+ which was considered unnecessarily high

F Electro mechanical dissociation, the effect of the mechanical activity on the influx (DMM) patterns for Na^{24} and Br^{8}

It seems quite possible that the mechanical activity can affect both the influx and efflux patterns by causing variations in the surface volume relationship during the contraction phase (see chapter I) This in its turn might give rise to mechanical artifacts by 1) extracellular squeezing and 2) squeezing of the extra cardiac surface layer (cf general discussion) Electro mechanical dissociation would give possibilities for studying the effect of the mechanical activity on the influx patterns for Na^{24} and Br^{8}

Electro mechanical dissociation has been described *inter alia* by ANTONI ENGSTFELDT and FLECKENSTEIN (1960) for frog ventricle muscle by reduction of the Ca^{++} content of the soaking solution WINEGRAD (1961) discusses the importance of the Ca^{++} ions in an electro mechanical coupling

According to WEIDMANN (1955) reduction in Ca^{++} concentration does not affect the resting potential different Ca^{++} concentrations varying from ¼ to 4 times the normal did not affect slow depolarization in a pace maker region (in this concentration range a change of frequency is only produced by a change in the threshold potential)

A low calcium environment does not only affect the electro mechanical coupling WEIDMANN (1955) with a voltage clamp technique showed that the Na permeability during depolarization was reduced if the calcium concentration was lowered

Experiments were made on isolated spontaneously beating toad auricles mounted as described for the sampling method (chapter V B a) The ECG was recorded in the usual way and the mechanical activity (mechanogram) as described in chapter IV A b In some cases the action potential was also recorded with a suction electrode The results of such an experiment are shown in Fig 40 where all the action potentials were measured with an electrode which was neither moved nor released from the tissue during this period It can be seen that the amplitude of the action potential decreases with sucking

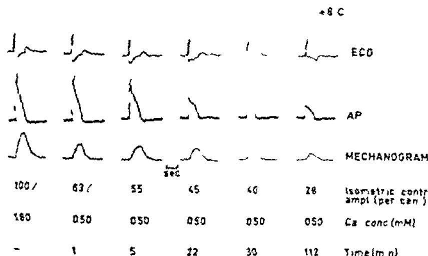


Fig 40 Recordings of ECG, action potential (suction electrode) and mechanogram (isometric) on a spontaneously beating atrium. The isometric amplitude after bathing for different periods in a solution containing 0.5 mM Ca^{++} is given

time' in accordance with the findings of SCHÜTZ (1931) A new sucking after about 115 min from the beginning of the experiment gave an action potential which had about the same amplitude as that of the first in Fig 40 The mean amplitude of the isometric contractions with perfusion of Ringer solution containing 0.5 mM Ca^{++} (solution I with reduction of the Ca^{++} concentration) was for six atrial preparations 25.4 % (range 18.5–27.5) of that with 1.8 mM Ca^{++} In all cases the electro mechanical dissociation was reversible In one case perfusion was made with a Ringer solution of 0.4 mM Ca^{++} which induced a reversible reduction of the contraction amplitude to 15.5 % of the original value In two preparations perfused with 0.25 mM Ca^{++} the mean amplitude was reduced to 9.9 % but subsequent perfusion with 1.8 mM Ca^{++} did not fully restore the contraction amplitude All experiments were made at a temperature of +8°C

Reducing the calcium concentration from 1.8 to 0.5 mM Ca^{++} usually lengthened the duration of the action potential by 10 % The time relations of the contraction cycle (start and maximum contraction) to the ECG and action potential were the same with normal and low calcium concentrations

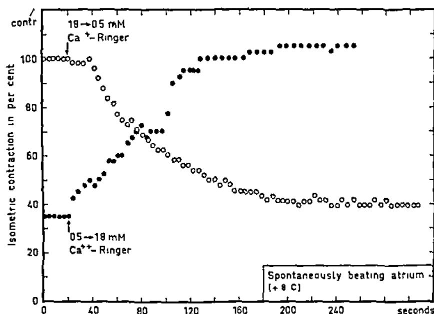


Fig 41 Isometric contraction amplitude (in per cent of that obtained in 1.8 mM Ca^{++}) of a spontaneously beating atrium in a Ringer solution containing 0.5 mM Ca^{++} (open circles) When the concentration was restored to 1.8 mM Ca^{++} the contraction amplitude returned to its original level (solid circles) The recovery curve has been drawn on the same time scale as the first curve though in fact it was subsequent to it

Fig 41 shows the electro mechanical dissociation for a spontaneously beating atrium during perfusion with a Ringer solution containing 0.5 mM Ca^{++} the amplitude of contraction at 1.8 mM Ca^{++} has been taken as the 100% level Perfusion was begun with 1.8 mM Ca^{++} and then suddenly switched to 0.5 mM Ca^{++} later when the contraction amplitude value had been steady for some time the perfusion was switched back to 1.8 mM Ca^{++} The results allow an approximate estimate of the equilibration rate for calcium in the preparation the value was similar to that found by NIEDERGERKE (1959)

Preliminary studies indicate that the electro mechanical dissociation induced by reduction of the Ca^{++} concentration is similar in sinus venosus

No *influx* experiments have as yet been made on the preparation with reduced amplitude of contraction and therefore the effect of the

mechanical activity on the extra cardiac surface film cannot be assessed *directly*. Other experiments which give *indirect* information were made with a contraction amplitude of only 25% of the normal; these experiments will be described later (see efflux experiments Fig. 46 a–c pp. 107–109).

VIII *The efflux of tracer from the sinus venosus and atrium under different perfusion conditions*

Some of the literature relating to ion fluxes during single action cycles in heart muscle was reviewed in the introduction. It has been claimed that K^{+} efflux shows cyclical variations in relation to the heart cycle (WILDE and O'BRIEN 1953, DANIELSON, ÖBRINK and SJOSTRAND 1961, 1962, LORBER *et al.* 1962); similar results have also been obtained for Na^{+} efflux (DANIELSON, ÖBRINK and SJOSTRAND 1961, 1962). The common working hypothesis in these experiments has been that the radioactive pattern in a perfusate reflects the ion flux between the intra- and extracellular spaces in the tissue, even if both damping phenomena and time lags make the results uncertain and difficult to interpret (the type of preparation and perfusion technique used often make it difficult to compare results). In all the works discussed there is the possibility that the efflux patterns resulted from mechanical activity. A mechanical "squeezing" effect has been considered very unlikely on *indirect* grounds, but *no direct* proof that the ion flux pattern is independent of mechanical activity has been forthcoming.

To obtain optimal conditions in an efflux experiment with the "wash-out" technique, it is a requirement that the movement of tracer across the boundary between the preparation and the perfusion solution is not the rate-limiting step. *Perfusion of the preparation must therefore be arranged so that the unstirred layer which surrounds it is as small as possible* (in those cases where the preparation has a mechanical activity, the unstirred layer should in addition not vary significantly in relation to the mechanical activity phase). *A turbulent flow with a high and constant linear mean velocity should therefore be expected to give the best distribution conditions between the preparation and the perfusion solution.*

In order to study the importance of the perfusion conditions for

the distribution kinetics of the tracer between the preparation and the perfusion solution the relationship between perfusion velocity and the efflux pattern has been studied

The movement of tracer from the non beating preparation to the perfusion solution at different perfusion velocities

Fig 42 shows the results obtained on a non beating sinus venosus (the preparation did not beat at a perfusion temperature of +5 C) the method used was that described in chapter V B a The sinus venosus was loaded in radioactive Ringer solution (Na^{24} Br^{82}) for one hour at room temperature with continuous vibration stirring (at this temperature it beat spontaneously) Two efflux experiments were made on the same preparation after loading in the radioactive Ringer solution (see chapter V B a) The first experiment (curve I in the figure) was made with a low perfusion velocity (about 0 8 g perfusion solution per sec corresponding to $v \approx 35$ cm/sec) After a second loading in the radioactive Ringer solution (1 hour) the second efflux experiment (curve II) was made at a high perfusion velocity (about 6 g perfusion solution per sec corresponding to $v \approx 265$ cm/sec) Fig 42 shows the total amounts and concentrations of Na^{24} and Br^{82} in the perfusate (the analysis accuracy for Na^{24} and Br^{82} were the same) and also the amount of perfusate in each individual sample The ratio between the total Br^{82} and Na^{24} in each sample is given in the figure *In the discussion which follows the assumption is made that the specific activity of the preparation was the same at the beginning of the two efflux experiments*

As expected the concentrations of Na^{24} and Br^{82} in the perfusate were higher with a low than a high perfusion velocity The middle section of the figure shows that the rate of efflux of the tracers was greater during a perfusion at the higher velocity (amount = concentration times sample volume) At both perfusion velocities the ratios between the total amounts of Br^{82} and Na^{24} in each sample were relatively constant (this shows that the relative depletion of the two isotopes was the same during the period of the experiment) at the higher velocity however there was a somewhat higher Br^{82}/Na^{24} ratio in the perfusate

Fig 43 shows an efflux experiment made on a non beating sinus venosus which was loaded for one hour at room temperature with

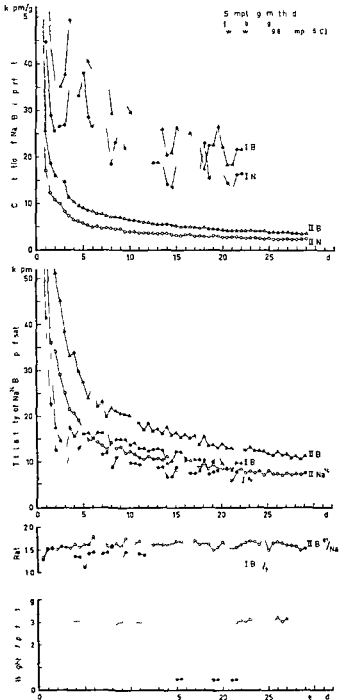


Fig 4. Two efflux experiments for Na[24] and Br[82] on a non beating sinus venosus by the method described in chapter V B a The accuracy of the analysis was for Na better than ±2 % Br[82] better than ±1 % The pre

vibration stirring with the same active Ringer solution as was used in the experiment of Fig. 42. The perfusion velocity was held constant (about 1.8 μl perfusate per sec corresponding to $v \approx 50$ cm/sec) for 15 seconds when it was stopped for 15 sec. Perfusion was then restarted at the same velocity which was then gradually reduced (note the rising concentrations of the isotopes in the second part of the experiment). The time courses of the effluxes (amount) of the tracers during the first perfusion period are similar to those of curve II in Fig. 42. The rest of the experiment is interpreted as follows. During the 15 seconds when the perfusion was stopped, outflow of the tracers into the stationary solution surrounding the preparation continued; thus the amounts of Na^{24} and Br^{82} in the perfusate when the perfusion is recommenced are initially high but very soon return to about the same level as just before perfusion was stopped. The Br^{82}/Na^{24} ratio diminished as the perfusion velocity was reduced during the second half of the experiment (cf. experiment of Fig. 42). Whether there is actually a ratio change in relation to the perfusion velocity is difficult to assess, but it seems plausible. Changes in relative depletion would give a varying ratio but this can be excluded (cf. page 97, the experiment of Fig. 42). The concentration of Na^{24} and Br^{82} in the perfusate shows that if the perfusion volume is below a certain critical level (after the time of 20 seconds) the tracer concentration rises even though the total amount in each sample is constant or diminished. *Small variations in the perfusion velocity for this reason may thus result in large fluctuations of the concentration in the perfusate.*

In the experiments of Figs. 42 and 43, the same active Ringer solution was used for loading the preparations, which facilitates certain comparisons between these experiments (further experimets are desirable). It seems probable from the experiments of Figs. 42 and 43 that the perfusion velocity is a factor affecting the rate of efflux of the tracer from the preparation. It is also probable that the nature of the ion itself is important since the ratio between the amounts of Br^{82} and Na^{24} in the perfusate were also dependent on perfusion veloc-

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paration was soaked for 1 hour at room temperature with vibration stirring in a loading solution containing 2117 kcpm Na^{24}/mg, 1870 kcpm Br^{82}/mg, i.e. $Br^{82}/Na^{24} = 0.86$ (1 kcpm = 10^3 cpm). The corresponding ESA for the active Ringer solution was 2550 kcpm Na^{24}/μmole Na and 6631 kcpm Br^{82}/μmole Br.

The figure shows the concentrations and total amounts for Na^{24} and Br^{82} in the perfusate. The volumes of perfusate samples are shown in the lower part of the figure (there were 2 samples per second) for the two efflux experiments (I and II) made on the same preparation (each after loading for 1 hour).

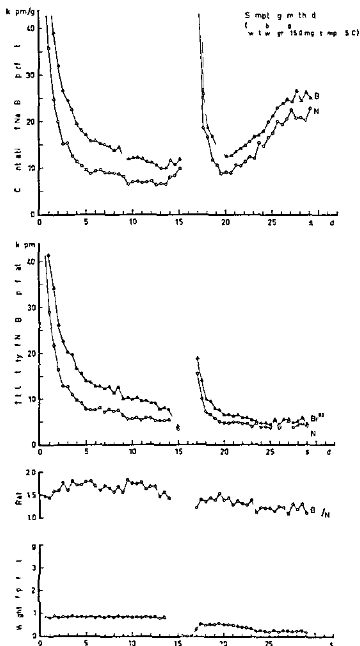


FIG 43 Efflux of Na^{24} and Br^{82} from a non beating sinus venosus (method described in chapter V B a) For accuracy of analysis see legend of Fig 40 The preparation was loaded for 1 hour at room temperature with the same active Ringer solution as in the experiment of Fig 42 (see figure text) The figure shows the concentrations and total amounts of Na^{24} and Br^{82} and the Br^{82}/Na^{24} ratios in the perfusate samples The lowest curve shows the weights of the sample of perfusate (2 samples per second) Perfusion was stopped between 15 secs and 16.5 secs (1.5 secs) and then recommenced at almost the initial rate which was then progressively decreased (see text for further details)

ity It seems most probable that the lower ratio obtained with lower perfusion rates depends essentially on a reduction in the efflux rate of Br^{82} (cf the amounts of Br^{8} and Na^{24} in the experiment of Fig 42 between the times 9–13 sec at both velocities cf also the values in the perfusate of the experiment of Fig 43 at 10 and 24–26 sec) The variations in tracer concentration in the perfusate were greater with lower perfusion velocities *At low perfusion velocities both the low velocity itself and the unstirred layer may become rate limiting steps* in determining the movement of the tracer from the preparation into the perfusion solution

When the tracer moves from the tissue into the convection stream of the non radioactive Ringer perfusant it has to pass through the unstirred layer surrounding the preparation This unstirred layer can schematically be regarded as a compartment lying between the tissue and convection stream compartments The volume of the tissue can be regarded as constant while that of the convection stream of inac tive Ringer solution depends on the perfusion velocity through the system the greater the velocity the larger the volume *The volume of the unstirred layer compartment depends to some extent on the perfusion velocity* (cf chapter VII B) in that at higher velocities it is reduced According to HARRIS (1960 p 148) the diffusion is con siderably slower in the extracellular space than in free solution because of restriction in the tissue matrix Ionic diffusion in an unstirred layer surrounding a biological preparation may also be modi fied compared with that in free solution (cf HALE and REICHEN BERG 1949 HELFFERICH 1959)

The total activity ratio Br^{8}/Na^{24} which was dependent on the perfusion velocity (experiments of Figs 42 and 43) can be interpreted in terms of the effects of an unstirred layer As is evident in Fig 42 and particularly in Fig 43 the concentration of the tracer is inversely related to the perfusion velocity At a high velocity the concentration gradient across the boundary between the unstirred layer and the convection stream of non radioactive Ringer solution is greater than with a low perfusion velocity At high velocities therefore the differ ences in mobility become more evident because the transport pro cesses are further away from the equilibrium state Thus since the Br^{-} ion has a mobility which is about 14 times that of the Na^{+} ion (cf ROBINSON and STOKES 1955) the Br^{8}/Na^{24} ratio should increase with increasing perfusion velocity

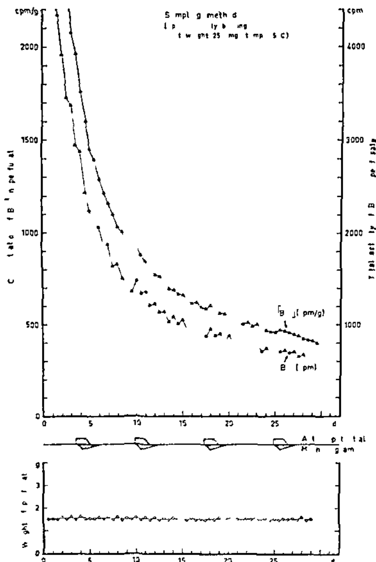


FIG 4 Efflux of Br⁸² from a spontaneously beating sinus venosus (chapter V I a) Accuracy of analysis was ± 1 The sinus venosus was loaded for 1 hour at room temperature with vibration stirring in an active Ring r solution containing 11 0 kcpm Br⁸² mg (ESA = 412 kcpm Br⁸²/μmol Br) (1 kcpm = 10³ cpm) The figure shows the concentration (left ordinate) and total amount (right ordinate) for Br⁸² in the perfusate samples (sample volume given below ~ samples per second) The electrical and mechanical activities are shown schematically (see also text)

The mechanical activity as discussed in chapter VII I *may affect both the observed influxes and effluxes by producing variations in the surface volume relationships* Such variation could give rise to mechanical artifacts in two ways 1) *Extracellular squeezing resulting in expulsion of extracellular fluid into the perfusion solution* may occur A squeezing effect might also alter the dimensions of the small extracellular spaces and in this way affect the physical chemical properties e g for diffusion 2) *The changing form of the preparation during contraction may distort the extra cardiac surface layer* In an efflux experiment this surface layer corresponds to an unstirred layer which thus may undergo variations during the mechanical cycle The only certain way to exclude these possibilities would be to study simultaneous fluxes of different tracer ions of which one could be safely assumed to be essentially extracellular The SO_4 ion fulfils the later role the heart muscle cells are relatively impermeable to it (cf JOHNSON 1955 PAGE 1962 NIEDERGERKE 1963 DANIELSON 1964) This ion can also be obtained with high specific activity of S^{35}

An efflux experiment for Na^{24} Br^{82} *and* $S^{35}O_4$ *was made on a spontaneously beating sinus venosus* (see Fig 46*a*) with a perfusion velocity corresponding to that used in the influx experiments by the DMM[1] At this perfusion velocity (7 µl of perfusion solution per sec corresponding to $v \approx 300$ cm/sec) the extra cardiac unstirred layer is very small and not likely to be rate limiting for the movement of tracer between the preparation and the perfusion solution (cf the summary of chapter VII B) As can be seen in Fig 46*a* there were no certain variations in the total activity or concentration of Na^{24} Br^{82} or $S^{35}O_4$ in the perfusate correlated with the electro mechanical cycle It is evident further that variations in the efflux of the three isotopes are clearly parallel

In order to study the effect of the mechanical activity an efflux experiment was made on the same preparation as in Fig 46a after soaking for 1 hour in radioactive Ringer solution containing only 0 5 mM Ca^{++} (the specific activity of Na^{24} Br^{82} and $S^{35}O_4$ in the Ringer solution was the same as in the experiment of Fig 46*a*) *The contrac*

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[1] Na^{24} and Br^{82} were determined by gamma spectrometry as described in chapter IV C a 2 The 0 16 MeV beta emission of the S^{35} (no gamma emission) was absorbed by the aluminium sheath of the NaI(Tl) crystal S^{35} was determined after decay of the Na^{24} and Br^{82} by determination in a liquid scintillation counter as described in chapter IV C b

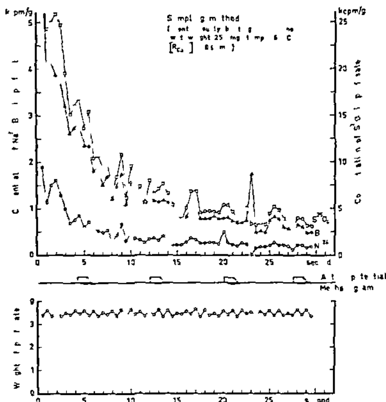


Fig 46 *b* (legen l p 106)

statistical counting error (cf chapter VI p 53)] The result is in agreement with other efflux experiments for Na [+] and Br[8] on sponta neously beating sinus venosus preparations with perfusion velocities corresponding to that used in the influx experiments (DMM) i e a mean linear velocity of 300 cm/sec

If extracellular squeezing were an important factor in determining the efflux pattern this should also be observed in an experiment when the perfusion conditions are comparable to those in the experiments of Fig 46a–c In these experiments the extra cardiac unstirred layer is pre umably small and contact between the preparation and the perfu ion solution particularly good

Fig 46*a–c* show al-o one of the *weaknesses of the efflux method* Only four cardiac cycles could be studied (longer efflux experiments have al-o been made but have not essentially augmented our know ledge) which together with the condition that the specific activity

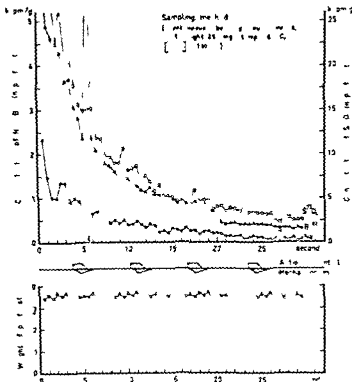


Fig 48 c (legend p 106)

of the preparation is in a transient state make it *impossible to use a type of continuous averaging in order to eliminate the background noise*

Only in those cases where the perfusion conditions could be regarded as poor were the variations of total activity and concentrations of the isotope in the perfusate correlated with the electro mechanical events This suggests that *the mechanical activity causes variations in the external unstirred layer which at low mean linear perfusion velocities becomes an important buffer compartment between the tissue and the inactive Ringer solution* Efflux of tracer occurs continually from the tissue into this buffer compartment and if it varies in size in relation to the mechanical cycle (changed surface volume relationship) so that for example its volume is reduced with a contraction this will be manifested in the perfusate as an increased quantity and concentration of tracer *It is unlikely that such small volume fluctuations could be recorded even by weighing the perfusate or using a*

radioactive volume indicator At higher perfusion velocities when the extra cardiac unstirred layer is reduced the effect of the mechanical activity is also presumably greatly reduced

Summary Efflux of tracer from the tissue to the convection stream perfusing non radioactive Ringer solution must occur through the unstirred layer which surrounds the preparation under these conditions With a low perfusion velocity it seems likely that an unstirred layer may be a rate limiting step for the efflux of tracer from the preparation Evidence was obtained suggesting that the perfusion velocity affected the efflux kinetics and that different ions were affected quantitatively differently In the efflux experiments with spontaneously beating preparations it is only when the perfusion conditions could be described as poor that there were variations in the total activity and concentrations of the isotopes which could be correlated to the electro mechanical cycles *If an extracellular squeezing were an important determinant of efflux kinetics this effect should also be observed when the perfusion conditions are comparable to those in an influx (DMM) experiment (Fig 46a–c) The results obtained suggest rather that distortions in the extra cardiac surface layer occur if these become evident in the efflux pattern they would be expected to be correlated with the mechanical activity* See further general discussion

As discussed in chapter I there are only a few reports in the literature of direct studies of the time relation between ion fluxes and the electro mechanical activity of the heart tissue. WILDE and O'BRIEN (1953), DANIELSON, ÖBRINK and SJÖSTRAND (1961, 1962) and LOUBERT et al. (1962) studied the K^{42} efflux from isolated heart preparations ("wash out" technique). DANIELSON, ÖBRINK and SJÖSTRAND (1961, 1962) studied also Na^{24} efflux. Preliminary results on the Na^{24} influx (sinus venosus) have been reported by SJÖSTRAND (1962, 1963).

In the present work the efflux from spontaneously beating sinus venosus and atrium has been studied with the help of radioactive isotopes with a "wash out" technique. The radioactive influx has also been studied with a new perfusion and analysis technique referred to previously as the "direct monitoring method" (DMM). By perfusion with a high linear mean velocity ($v \approx 300$ cm/sec) the efflux of Na^{24} and Br^{82} did not exhibit any fluctuations which could with certainty be correlated with the electro mechanical cycles of activity of the spontaneously beating preparation. In influx experiments made by the DMM fluctuations related to the electro mechanical events of the heart cycle were obtained for both Na^{24} and Br^{82}. The methodological problems associated with these experiments were discussed in chapters VI, VII and VIII.

The complexity of the tissue preparation used

An isolated heart muscle preparation is both structurally and functionally a particularly complex system. It is multi cellular and consists structurally of several tissue compartments (a classification into only intracellular and extracellular spaces appears in practice to be an over simplification). From a functional aspect also the concept of only two compartments, intra- and extracellular, appears to be insufficient. The heart muscle cell differs from the much studied nerve axon in having in addition to the resting and action potentials a mechanical activity. It is not easy to dissociate the electrical activity (action potential) and the mechanical activity (contraction) which

run rather in parallel Studies of a tracer flux in a preparation with such a complex structural and functional constitution cannot *a priori* be expected to give results which indicate a simple ion flux pattern in terms of only intra and extracellular compartments[1]

General views regarding tracer measurements on heart tissue

The use of radioactive isotopes in the determination in the resting state of the ionic movements between the intra and extracellular spaces presents varying degrees of difficulty A measured tracer flux can in general be said to depend on at least two main factors the exchange relationships between the cells and the extracellular space and between the latter and the perfusion solution Measurements of the K^+ flux are therefore the simplest since the intracellular concentration of this ion is high The difficulties involved in the determination of Na^+ and Cl^- (Br^-) fluxes are considerably greater since their intracellular concentrations are rather low The determination of the sodium exchange between cells and extracellular space (and probably also Cl^- exchange) seems at present possible only by using extremely short time constants Even if the analysis technique can give from the time aspect good resolution the results obtained are difficult to assess if damping phenomena and time lags in the system studied can not be calculated accurately (the extracellular volume should for these reasons be small compared to the intracellular compartment)

The temperature effect on electrolyte distribution and fluxes must also be considered HAAS and GLITSCH (1962) found that the K^{42} efflux from non beating frog atria increased with temperature Studying the temperature effect on electrolyte exchange in cat heart papillary muscle PAGE GOERKE and STORM (1964) found no changes in cell K and Na contents in mammalian papillary muscles cooled to 17–18 C with further cooling there were marked losses of cell K and increases in cell Na Cl and water However cooling of frog heart muscle from room temperature to + 5 C is not analogous to cooling mammalian heart tissue to a similar extent in relation to the normal temperature (cf the results for K^{42} efflux from frog atria by HAAS and GLITSCH cited above)

[1] The kinetics of ion exchange between different phases in a multicompartment system have been treated by HAAS (1962)

Possible mechanical influences on tracer kinetics in "wash-out" experiments on heart muscle

Any effects on the tracer flux induced by mechanical activity would most likely be correlated with the contraction cycles. For instance it seems not unlikely that the surface volume relationships of the tissue preparation will be altered during mechanical changes. As pointed out in chapter VIII mechanical "squeezing" may have two main effects: 1) the expulsion of extracellular fluid and 2) mechanical distortion of an unstirred layer surrounding the preparation during perfusion ("external squeezing" effect). The results reported in chapter VIII do not exclude the possibility of an "extracellular squeezing" and it is probable that under certain perfusion conditions an "external squeezing" effect becomes a limiting factor. It must therefore be borne in mind that when ion effluxes as studied by the "wash out" technique appear to be correlated with the events of the heart cycle the question of an "external squeezing" effect should be excluded.

The release of K^+ from a perfused turtle heart

By perfusing the coronary system of a K^+ loaded turtle heart, WILDE and O'BRIEN (1953) (see also WILDE 1957*a*, *b*) found cyclical variations of the K^+ efflux (correction for the transport time and boundary spreading due to the long distance from the capillaries in the preparation to the point where the perfusate was collected were made). The perfusion volume was about 0.03 ml per second and was determined directly by adding I^{131} labelled albumin or $P^{32}O_4$ (the flow was constant between contractions but during systole a "squeezing" effect occurred, the volume collected increasing three fold; the authors believed that this was due to the veins being emptied as when a sponge is squeezed). A corrected K^+ concentration was calculated by adjustment for volume changes in flow; these values exhibited a maximum immediately after the T wave in the electrocardiogram. — It should be pointed out that the perfusion volume in these experiments was small, subject to large fluctuations. With the technique used for the determination of the perfusion volume, corrections for volume fluctuations which might be caused by changes in the intravascular buffer volume induced by the mechanical activity could not be calculated. The intravascular buffer volume can be conceived as an unstirred layer (corresponding to the immobile layer in the

plasmatic zone discussed by COPLEY, SCOTT BLAIR, BALE and STAPLE 1960) which could almost certainly be affected by the changing perfusion flow in the vascular system (cf. chapter VIII, p. 99).

Efflux of Na^{24} and K^{42} from the isolated frog heart

DANIELSON, ÖBRINK and SJÖSTRAND (1961, 1962) found cyclical variations of Na^{24} efflux when an isolated frog heart preparation (sinus venosus, atrium or sinus atrium) was perfused. Because of the difficulty of obtaining sufficiently high specific activities of K^{42} only in one efflux experiment on the sinoatrial preparation was a sufficiently high activity of K^{42} obtained. Movements of Na^{24} and K^{42} studied simultaneously showed cyclical variations in relation to the recorded electrical activity (the ratio between Na^{24} and K^{42} in the perfusate did not show any definite variations in relation to the heart cycle). The perfusion velocity was between 0.1 and 0.5 ml/sec (variations of $\pm 10\%$) corresponding to a mean linear velocity of $\bar{v} \approx 5$–22 cm/sec. The perfusion flow should therefore have been within the region where the tracer efflux should be expected to show large concentration variation in response to small changes in the perfusion velocity (cf. Figs. 42, p. 98 and 43, p. 100).

Efflux of K^{42} from frog ventricle

LOPBER *et al.* (1962) perfused a frog ventricle preparation and found cyclical variations in the total amount of K^{42} in the perfusate (there was a maximum during and of approximately of the same length as the action potential). The possibility that the efflux pattern observed was caused by mechanical activity was excluded by the authors because of the change in the appearance of the efflux peak (lower and broader) when the perfusing fluid was changed from normal to potassium free Ringer. An immediate and marked increase of the isometric tension occurred and lasted for several minutes when the normal Ringer was replaced by the potassium free solution; the authors considered that reduced contractility would alter the form of the efflux peaks for mechanical reasons. On the other hand perfusion with potassium free Ringer solution resulted in a marked decrease in the efflux rate for K^{42}. The perfusion velocity in these experiments was about 0.17 ml/sec (corresponding to a turn over of 20 times per second of the free fluid volume surrounding the tissue in the perfusion chamber). From the data given it can be calculated that the

mean linear perfusion velocity was not greater than 50 cm/sec and thus the conditions in comparison with those in the system used in the experiments reported in chapter VIII are most nearly comparable with those in the experiment of Fig. 42 p. 98 (experiment I) Fig. 43 p. 100 (first period of efflux experiment) and Fig. 45 (p. 104). It seems therefore likely that in the experiments of FORBES et al. (1962) there was a significant extra cardiac buffer volume and therefore an "external squeezing" effect is a possible alternative explanation for the K^{42} pattern they obtained. The increased isometric tension after change over to a potassium free Ringer solution does not exclude an "external squeezing" effect since the total efflux of K^{+} was strongly reduced at the same time. The movements of K^{+} across the external surface layer could therefore be expected to be considerably slower; the concentration of K^{+} in this layer would therefore be estimated to be lower on perfusion with potassium free Ringer solution whereby in spite of increased mechanical activity the efflux peak for K^{42} would be reduced.

Direct monitoring method (DMM)—advantages and limitations

As pointed out in chapter VIII one of the weaknesses with the "wash out" technique is that continuous averaging cannot be performed. With an influx experiment according to the direct monitoring method (DMM, see p. 45) the conditions are considerably more favourable for an analysis of the tracer flux because continuous averaging is possible. This means that although individual measurements may be regarded as uncertain and even exhibit such small variations as to lie within the error of the method they can collectively be used to derive useful information (continuous averaging is discussed in chapter VI). *In the DMM continuous averaging over a large number of cycles may reveal systematic variations in the tracer flux.* If the perfusion conditions during the whole experiment are the same (constant relationships between the lengths of the Ringer and silicone oil perfusion periods) the variations in the analytical and perfusion systems will have the character of "random fluctuation noise".

DMM also has a rather good time resolution. It was pointed out in chapter VII C (p. 83) that theoretically the mean cycle obtained by the continuous averaging technique can exhibit a time shift

corresponding maximally to $-(t_r+t_s)$ msec in relation to the reference point (=e g the ECG recording) and thus the correction range is known The resolution with respect to time in the DMM is therefore determined by how short t_r (the Ringer perfusion period) and t_s (silicone perfusion period) can be made these are technical matters[1]

If the DMM is made when a uniform specific activity has been obtained (the specific activity of the preparation = that of the perfusing active Ringer solution) it offers advantageous conditions for studying net fluxes between the preparation and the perfusing solution What information these net fluxes can give depends on the properties of the object studied experiments on heart muscle preparations must give less clear results than studies of non contractile preparations A tissue consisting of a single cell or cell groups without an extracellular space (tissue culture) also makes it easier for direct studies of any transmembrane net fluxes It should however be pointed out that DMM can only give information about the flux of the tracer into the preparation as a whole (amount per unit time) and so long as there are internal concentration gradients for the specific activity (uneven specific activity) estimates of the flux in pmole/cm² or mmole/kg of tissue per beat cannot be made

General comments on the influx experiments (DMM) performed

The experiments so far performed with DMM have been made essentially to demonstrate the technique and to investigate its potentialities The resolution in the analysis method (signal/noise ratio) can in general be expected to be greatest when the signal/noise ratio is highest since the background is much greater than the signal in the preparation the resolution is rather poor It is therefore advantageous to study the first part of the tracer influx (upstroke of the DC component) even if the later part (plateau of the DC component) could be expected to better reflect the tracer influx into the cellular space[2] Only in one of the influx experiments so far performed by

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[1] In the experiments made with the DMM up to now t_r was ≈100 msec and t_s ≈ 1000–1500 msec

[2] As pointed out earlier (p 8) it can be expected that there are several components in the tracer influx (a rapid fraction corresponding to the extracellular space and one or more less rapid components probably corresponding to the intracellular space see further chapter VII D p 84)

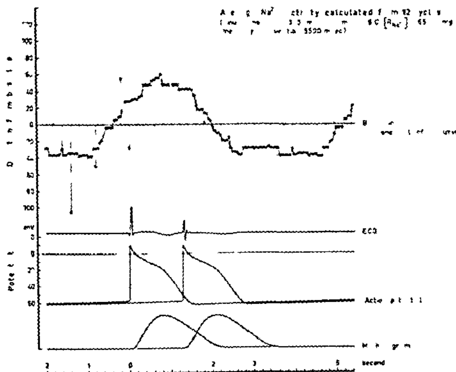


Fig 47 A mean cycle for Na activity calculated by the e-a t method for the first influx experiment of Fig 24 (p 5) The base line corresponds to the mean trend line (described in chapter VI p 54) The ECG is drawn from the original recording while the action potential and mechanogram are drawn from other experiments on the same type of preparation (chapter II c p 1") The open circles are the medians of the deviation from the base line of the original measurements occurring in each 100 msec interval (original value method) The solid circles are the median of 1 intercept interpolations values for each 50 msec interval (intercept interpolation method) The standard deviation for the mean values are similar to those given for the experiment of Fig 2f (p 56) in chapter VI (p 57)

The maximal uncertainty in the positions of the radioactivity pattern in relation to electromechanical activity is $(t + t)$ In this case a shift of $(t + t) =$ (105 + 1195) = 1300 msec to the left (Each measurement is arbitrarily assigned to the end of the period whose radioactivity is measured the most correct value must therefore lie somewhere within the interval of this period i e not more than $(t + t)$ to the left of where it is arbitrarily placed Cf p 83) Wet weight of preparation 19 3 mg Calculated O_g = 1 6 mg calculated trapped volume = 0 7 mg Perfusion and analysis programme (DMM) t = 105 msec t = 1195 msec t_m = 958 msec Active Ringer solution ESA 1 48 kcps Na /μmol Na (solution I) (kcps = 10^3 cps)

DMM was an equilibrial level obtained (the experiment of Fig 25 p I) In calculating the mean cycle the relation of the AC component to that of the DC component was calculated during a period when the DC component was not steady since a uniform specific

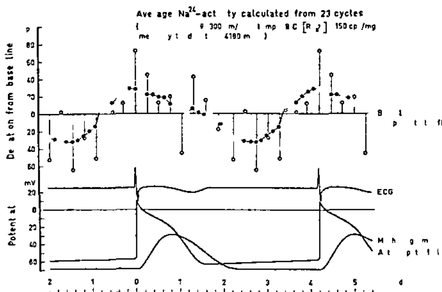


FIG 48 A mean cycle for Na^{24} activity calculated by the *c a t* method from 23 cycles in an influx experiment (DMM) on a spontaneously beating sinus venosus The base line corresponds to the mean trend line (described in chapter VI p 54) The ECG is drawn from the original recording while the action potential and mechanogram are drawn from other experiments on the same type of preparation (chapter II c p 17) The open circles are the arithmetic means of the deviations from the base line of the original measurements occurring in each 200 msec interval (original value method) The solid circles are the arithmetic means of 23 intercept–interpolation values for each 130 msec interval (intercept–interpolation method) The standard deviation for the mean values is similar to that given for the experiment of Fig 26 (p 56) in chapter VI (p 57)

The maximal uncertainty in the positions of the radioactivity pattern in relation to electromechanical activity is in this case a shift of $(t + t_s) = (120 + 1195) = 131$ msec to the left (cf legend Fig 47) Wet weight of preparation 19 7 mg Calculated $O_R = 1\ 7$ mg calculated trapped volume = 0 7 mg Iter fusion and analysis programme (DMM) $t = 120$ msec $t_s = 1195$ msec $t_m = 1025$ msec Active Ringer solution ES V 1 35 kcps Na^{24}/μmole Na (solution I) (kcps = 10^3 cps)

activity had not been attained Whether it is correct under these conditions to determine a mean cycle can naturally be discussed (cf above) As mentioned in chapter VII C (p 75) it seemed permissible for technical reasons to calculate a mean cycle during the rising phase of the DC curve because the relationship between the *mean influxes* during the Ringer and silicone oil perfusion periods remained constant for the whole experiment

Two mean cycles for Na^{24}

With the reservations discussed above the mean cycle for two influx experiments with Na^{24} was calculated by the *c a t* method

described in chapter VI Fig 47 shows the recorded counted activity in relation to the base line (DC component = passive distribution plus activity base line) during the first of the two influx experiments illustrated in Fig 24 (for further information see chapter VI p 51) Fig 48 shows the counting activity in relation to the base line for Na^{24} calculated for 23 heart cycles during another influx experiment These two experiments for Na^{24} together with a Br^{82} experiment whose mean cycle is shown in Fig 26 (p 56) were from a technical aspect the most satisfactory up to now [1] The K^{42} influx during the single heart cycle has not been studied because of the difficulty of obtaining sufficiently high specific activity of this isotope (cf p 50) The sinus venosus preparation on which the result shown in Fig 47 was obtained with DMM had two electrical activities it cannot be assessed which of these two electro mechanical activities was the dominant (visual inspection revealed that the preparation did exhibit a double contraction)

General remarks on the Na^{24} and Br^{82} activity curves for three different heart preparations

From the results presented in Fig 26 (Br^{82} p 56) and Figs 47 and 48 (Na^{24}) it appears that the AC component in the influx experiment (DMM) on the spontaneously beating sinus venosus does contain systematic fluctuations which can be demonstrated with the *c-a* *t* procedure The mean cycles shown in these figures exhibit fluctuations in counting activity which are related to the electro mechanical activity A maximum of the recorded radioactivity for both Na^{24} and Br^{82} occurs together with or shortly after depolarization while a minimum is obtained during the diastolic phase When the slope of the activity curve for the mean cycle is positive this means a net increase of activity in the system and vice versa (when the slope is zero i e parallel with the base line the net flux is also zero) As pointed out in chapter VII C the systematic fluctuations about the exponential influx curve (base line) in a spontaneously beating preparation are

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[1] After the Br^{82} influx experiment of Figs 25 and 26 an influx experiment for Na^{24} was made on the same preparation this however failed for technical reasons (the background activity was not held constant during the experiment) The mean cycle for Na^{24} and Br^{82} calculated from results on the same preparation can therefore not be given

not necessarily evidence of ionic movements in or out of cells (cf al o chapter VIII p 105) The fluctuations may have other causes 1) The pattern of radioactivity in the tissue may depend on the relationship between the volume of the extra cardiac surface film (O_R) during the silicone oil perfusion and mechanical activity 2) The mechanical activity may cause alterations in the extracellular movements of the isotope in the tissue —If the variations in O_R were the *sole* cause of the fluctuations in activity calculated with the *c a t* method the pattern for *all* isotopes measured with the DMM should have essentially the same form and relation to the electro mechanical cycle independently of the relationship between t_r and t_s (this is discussed in chapter VII C pp 81 83) If the electro mechanical activity affected the movements of the isotope within the tissue this effect should be dependent to some extent on the relationship between t_r and t_s (cf chapter VII C pp 83 84) As discussed in chapter VII C (p 81) the limits for the correlation in time between the recorded electrical activity (ECG) and the calculated radioactivity pattern for the mean cycle can be determined One of the limits is shown in Figs 26 47 and 48 the other limit is maximally a shift (t +t_s) msec to the left of the radioactivity pattern in relation to the electro mechanical cycle

Can the tracer patterns for the mean cycle be explained by a change in O_R due to mechanical events?

If there is a correlation between the volume of the extra cardiac surface film O_R during silicone oil perfusion and mechanical activity then according to the arguments presented above the activity patterns should be the same for all isotopes (up to now only Na [1] and Br[8]) studied with DMM In all cases the activity patterns should be essentially similar in form and relation to the electro mechanical cycle this is not the case From the results described in chapter VIII concerning the efflux of Na [1] and Br[8] from a spontaneously beating preparation it is evident that distortion in a surface layer (external squeezing effect) is an important factor these variations occur in relation to the electro mechanical activity (maximal squeezing effect during the electro mechanical activity) If the volume of O_R was correlated to the electro mechanical activity it would therefore probably have a minimum during contraction and the recorded radioactivity pattern

for the mean cycle should therefore in this case show a minimum in connection with the electro mechanical activity This is not the case (cf Fig 26 p 56 and 48) It is difficult to attribute the results for the Na^{24} activity pattern in Fig 47 to the mechanical activity since the preparation exhibited a double contraction cycle *It appears therefore that variations in O_R correlated with the electro mechanical activity can be excluded as a cause of the alternating mean cycle radioactivity pattern calculated by the c a t method* (cf chapter VII C pp 81 83)

Can the tracer patterns for the mean cycle be explained by a mechanical distortion of the extracellular matrix (extracellular squeezing effect)?

If the activity pattern depends on a correlation between the tracer kinetics in the tissue and the electro mechanical activity the question of an extracellular squeezing must be discussed Extracellular squeezing may result in expulsion of extracellular fluid into the perfusion solution Such an effect will be expected to give a reduced radioactivity in the preparation during the mechanical activity Even if the radioactivity pattern in Figs 26 47 and 48 (see particularly Fig 26) is shifted ($t + t$) msec to the left *it is difficult to accept that the relationship of the radioactivity pattern to the mechanical activity can be explained as a result of extracellular squeezing* (cf also the summary of chapter VIII)

The possibility of there being a true biological explanation for the pattern obtained

If it can be excluded that the radioactive pattern of the mean cycle is not wholly mechanical in origin the possibility remains that both Na^{24} (Figs 47 48) and Br^{82} (Fig 26 p 56) oscillations arise from biological causes[1] More refined methods for example simultaneous studies of Na^{24} and Br^{82} on the same preparation should give more reliable information about this matter

As pointed out earlier the possibility of determining a transmembrane flux in a sinus venosus is very small because of the unfavour

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[1] The only way to exclude mechanical squeezing with certainty would be to do an experiment with DMM on a preparation with no mechanical activity (a reduced mechanical activity can be obtained on sinus venosus see chapter VII F)

able relationship between the volumes of the extracellular and intracellular compartments[1] and the great disparity in the electrolyte distribution. *It must however be pointed out that contact between the perfusion solution and the outermost cells of the preparation is particularly good under the conditions of a perfusion experiment by the DMM.* An example of this are the K^+ depolarization experiments described in chapter VII E. *It may therefore be possible that an ion flux between the extracellular and intracellular spaces was recorded since the measuring system used had a particularly good resolution with respect to time* (see above).

Even if it would be somewhat premature to attempt to analyse the present results in terms of current theories concerning ion fluxes and their relation to single electrical activity cycles, possible general mechanisms for the genesis of the results obtained may be considered.

1. Mechanical artifacts
2. Changes in "driving forces"
3. Changes in permeability
4. Changes in other unknown parameters

1. Of possible mechanical artifacts, variations in the extracardiac surface film (O_R) and deformation of the extracellular space due to "squeezing" can be excluded as causes of the results obtained for Na^+ and Br^-. One possibility which however cannot be neglected is that the mechanical activity of the preparation affects the extracellular matrix so that its physico-chemical properties are altered, *e.g. for diffusion.*

2. The cycles of electrical activity may be coupled with changes in the electro-chemical and electro-osmotic conditions within the excitable tissue. The "action potential" for example could be accompanied by reduced electro-osmotic forces which would result in an outward bulk flow (cf. TEORELL 1960). According to the electro-kinetic excitability analogue system described by TEORELL (1960, 1962) there is a "total net salt flux" which from the instant that depolarization begins is out of the cell and which just before or during repolarization changes to a gradually increasing influx. With the next depolarization it reverses again to an efflux (spontaneous repe

[1] DANIELSSON (1964) studied the SO_4 distribution in toad heart. The SO_4^{--} spaces were for sinus venosus 63 (±6.5), atrium 49 (±4.0) and ventricle 22 (±2.5) ml per 100 g wet tissue respectively.

titive excitation) These results have recently been confirmed by ionic tracer flux analysis on the "membrane oscillator" described above (TROBELL 1964)

3 Permeability changes associated with the electrical activity cycles seem a possibility According to HODGKIN and HUXLEY (1952 *a–d*) there should be a "sodium inward current" associated with depolarization caused by an increase in permeability for Na —As can be seen however in Figs 47 and 48 the radioactivity increase in the preparation (influx into the cells?) begins *before* depolarization It should be pointed out that the time relations between the electro mechanical activity and the radioactivity pattern recorded shown in Figs 26 (p 56) 47 and 48 represent only one of the boundary limits for this relationship (cf p 81) any shift of the radioactivity pattern should be to the left in the figures It is possible that the early inward sodium movement is connected with the spontaneous electrical activity of the sinus venosus one of the features of which is the slow depolarization of the pace maker and near pace maker regions (cf Figs 4 and 5 p 19)

The Br^{8} activity curve (Fig 26 p 56) for the "mean cycle" may also be regarded as a possible analogue for the Cl^{-} activity curve (cf p 50) As can be seen in Fig 26 the Br^{8} activity is maximal during the electrical activity rapidly decreasing during the steep slope of repolarization becoming minimal during most of the diastolic phase From potential and resistance measurements (Purkinje fibres) CARMELIET (1961) claimed that there is an appreciable inward current of chloride ions occurring directly after the crest of the action potential and persisting during the plateau The direction of the chloride ion movement during the slow diastolic depolarization was not clear but according to CARMELIET there were two possibilities 1 The chloride ions were moving inwards and exerted a stabilizing effect and 2 the chloride ions were moving outwards and favouring depolarization — As can be seen however in Fig 26 the radioactivity increase in the preparation (influx into cells?) begins *before* depolarization (cf time relation above) this being the case it may be connected with the spontaneous electrical activity of the sinus venosus The minimum value of the Br^{8} activity during the main part of the diastolic phase should in this case favour depolarization

An *explanation* of the radioactivity pattern for the mean cycle must however await further experimentation

GENERAL SUMMARY

The aim of the present work was to study the relationship between ion fluxes and the single electro mechanical cycle in the sinus venosus and atrium of toads.

Chapter I The purpose of the present investigation

A short discussion about the current theories for the genesis of resting and action potentials in excitable tissue together with a review of certain relevant experimental results in the literature are presented. The greater part of the works cited concern information about the ion fluxes per activity cycle obtained from studies of many cycles. Only in isolated cases has the flux during a single cycle been studied. There is as yet no uniform picture of the relation between ion fluxes and electrical activity based on solid experimental evidence. The main difficulty seems the lack of suitable experimental methods. The aim of the present work was to make a contribution to both the biological and methodological aspects of this problem. In order to study the direct relationship between the single electro mechanical cycle and the ion flux two main methods were used: an *influx method* and an *efflux method*.

Influx studies. The radioactive uptake by sinus venosus was measured discontinuously at short time intervals in a perfusion chamber placed in the well of a scintillation crystal. The preparation was alternately perfused with Ringer containing a gamma emitting isotope of the ion being studied (100 msec) and then with a hydrophobic silicone oil (1000 msec) which served to wash away the radioactive perfusing fluid so that the activity of the preparation could be determined. The electrical changes were followed continuously with a surface electrode (ECG). Heavy radiactive shielding was required.

Efflux studies. The heart preparation was loaded with radioactivity and washed by a stream of inactive Ringer solution ("wash out" technique). Two samples of the efflux were collected each second and good resolution in respect to time was obtained.

Chapter II The sinus venosus and the atrium of the heart of the toad

The macroscopic and microscopic anatomy and the electrical characteristics of the tissue are described For both influx and efflux studies the preparation was turned inside out so that the endothelial surface lay outmost and was bathed directly by the perfusing fluid In the ion flux studies made it was very important that the electrical and mechanical activities should be very nearly synchronous in all parts of the preparation Preliminary studies showed that at the low temperature used for the experiments the electrical activity could be regarded as being virtually synchronous throughout the preparation and that the ECG could be used as a reference point for determining the length of this activity The mechanical processes had a constant relationship to the electrical cycles

Chapter III The perfusion system

The perfusion system and the critical conditions required for the influx and efflux experiments are described It was found that for the influx experiments a mean linear velocity of about 300 cm/sec (perfusion volume 7 ml/sec) corresponded to about 400 changes/sec of the perfusion fluid around the preparation which had a wet weight of about 20 mg at this flow velocity both Ringer and silicone oil flows were strongly turbulent Turbulent flow was required both for good exchange properties between the Ringer and the preparation and for efficient removal of the Ringer during the silicone oil period

Chapter IV Methods

The recording methods (ECG action potential mechanogram and radioactivity) are described In the influx method (direct monitoring method DMM) great demands are made on the recording system since no individual measurement can be repeated Two separate analysis systems were therefore used 1) a continuous decimal pip system and 2) a discontinuously working digital printing system The accuracy of the radioactive analyses was adequate

Chapter V Procedure

Two types of influx methods were used 1) alternate perfusion of the preparation with Ringer and silicone oil and analysis of the radio

activity during the silicone period (direct monitoring method DMM) 2) determination of the amount of isotope or other test substance taken up from a soaking bath In the efflux experiments (sampling method SM) two samples of a perfusate which had flowed past a preparation loaded with the test substance or isotope were collected each second

Chapter VI Some influx experiments the calculations and the graphical representation of such experiments

Two influx experiments (DMM) one for Na [24] the other for Br[8] on isolated sinus venosus are described In both experiments the curves relating activity to time consist of at least two components 1) a mean exponential influx curve (DC component) on which are superimposed 2) oscillations (AC component) During one of the Na[24] influx experiments the sinus venosus stopped beating and the DC component then exhibited another time cour e (the rate of increase of radioactivity of the preparation decreased) The radioactivity was measured every 1 2–1 3 sec this is in relation to the biological process rather long and from this point of view the influx method can be regarded as rather coarse For this reason the radioactivity pattern differed greatly from cycle to cycle which was not unexpected because in addition to any systematic fluctuations the AC component (single measurements) also reflects all the random variations biological and non biological i e instabilities in the perfusion system and recording systems and statistical counting errors of the radioactivity In order to determine the true systematic pattern a type of continuous averaging known as the computing of average transients *c a t* was used The assumptions necessary in order to calculate a *c a t* are described together with the details of the procedure By means of this procedure it is possible to calculate a mean heart cycle and though certain reservations must be made this seems a useful procedure for extracting information

Chapter VII Some methodological problems of the influx experiments

1 *The background activity and trapped activity*

A sham influx experiment without a preparation in the perfusion chamber was made and the radioactivity pattern determined There are two sources of background activity 1) That arising extraneously from radioactivity in other parts of the perfusion system which could

not be entirely screened off and 2) that arising from some radioactivity that during silicone oil perfusion in the chamber is not been washed away This latter (trapped activity) was estimated to correspond to about 0 7 ml of active Ringer solution

B The surface layer of the heart preparation during Ringer and silicone oil perfusion

It is concluded that during Ringer perfusion the tissue is surrounded by an unstirred layer probably about 0 5–7 μ thick (this unstirred layer does not seem to be a rate limiting step in the movements of tracer between the Ringer solution and the tissues) During silicone oil perfusion following the passage of the Ringer silicone phase boundary an extra cardiac surface film of active Ringer solution (O_R) is left behind At the beginning of the silicone oil perfusion period this has the same specific activity as the Ringer solution (the volume of O_R was calculated to be equivalent to about 8 5 % of the wet weight of the preparation corresponding to a maximal thickness of 25–30 μ) The total radioactivity in the perfusion chamber during a single silicone oil period of about 1 second was practically constant

C The uptake of tracer in the tissue of the preparation during Ringer and silicone oil perfusion

Evidence is presented that the exponential increase of radioactivity with time in the preparation is representative for the mean activity in the tissue During the alternate Ringer silicone oil periods the tracer kinetics are different during Ringer perfusion the source of radioactivity is infinitely large while during the silicone oil perfusion period it is rather limited The course of the exponential curve depends on the electro mechanical activity of the tissue and the perfusion programme (ratio between the lengths of the Ringer and silicone oil perfusion periods) The AC component (mean cycle) exhibits systematic fluctuations in relation to the electro mechanical cycle of the spontaneously beating preparation These systematic fluctuations may possibly arise from non biological (biological = transmembrane ionic movements) in two ways 1) the mechanical activity may distort the external surface film (O_R) during silicone perfusion and 2) electro mechanical activity may produce changes in the extracellular space Further it is pointed out that a shift of the mean cycle with respect to time cannot be great (not more than 1 3 sec)

D The equilibration volumes and uptake rates during an influx experiment

The equilibration volumes were determined by soaking the tissue in a solution containing Na^{24} Br^{82} and raffinose the latter was used as an indicator for the extracellular space The uptake of raffinose in the sinus venosus and atrium was slower than that of Na^{24} and Br^{82} From these values it was concluded that during the influx experiments made with the DMM at a temperature of +5°C Na^{24} and Br^{82} moved primarily into the extracellular space (the equilibration time for the sinus venosus was about 200–250 sec during continuous Ringer perfusion) The influx of the isotopes in the DMM was faster than in a simple soaking technique

E Studies of the distribution of K^+ ions between the perfusion solution and the outermost cells of the tissue preparation

The time between the contact of the sinus venosus preparation with a Ringer solution containing 44 mM K^+ and the depolarization could enable an estimate to be made of the proximity of the outermost cells to the perfusion solution The time lag measured in this way thus was interpreted as the time required for the K^+ ions to reach the cellular membranes in sufficient concentrations to produce depolarization

F Electro mechanical dissociation the effect of the mechanical activity on the influx (DMM) patterns for Na^{24} and Br^{82}

Mechanical activity may possibly affect both influx and efflux studies by causing variations in the surface volume relationship of the tissue during the contraction phase These in their turn may generate mechanical artifacts by 1) altering the geometry of the extracellular space and 2) distorting a critical surface film surrounding the preparation Lowered mechanical activity without essentially altered electrical activity (electro mechanical dissociation) can be achieved by low Ca^{++} concentrations When the Ca^{++} concentration was lowered to 0 5 mM the contraction was then only 2 % of the normal amplitude (isometric) As yet no influx experiment has been made on such a preparation but the efflux has been studied

Chapter VIII The efflux of tracer from the sinus venosus and atrium under different perfusion conditions

To ascertain the importance of the perfusion conditions the relationship between *perfusion velocity* and the *efflux pattern* was studied in both spontaneously beating and inactive preparations It was found that the efflux depends on the perfusion velocity and is different for different ions Only when the perfusion velocity was low were cyclical variations in the amount and concentration of tracer in the *perfusate correlated to the electro mechanical activity of the spontaneously* beating preparation (depolarization caused increased efflux of tracer) At a high perfusion velocity (linear mean flow $\bar{v} \approx 300$ cm/sec) corresponding to the perfusion conditions of the influx experiments (DMM) there were no fluctuations in concentration or amount *in the perfusate related to the electro mechanical activity*

If extracellular squeezing were an important factor in the efflux this should also be seen with high perfusion velocities The results obtained rather suggest that the distortion of the unstirred surface layer surrounding the preparation is important such an external *squeezing effect would be expected to cause variations in the efflux* correlated with the mechanical activity One of the difficulties in obtaining clear cut results with an efflux method even under optimal perfusion conditions are that the duration of the experiment is severely limited as the radioactivity soon becomes reduced to unpracticable levels *Because of this a continuous averaging technique* as used for the influx experiments cannot be used to eliminate background noise

General discussion

With perfusion at a high linear mean velocity ($\bar{v} \approx 300$ cm/sec) there were no definite fluctuations in the efflux of Na^{+} or Br^{8} from a *spontaneously beating* heart preparation which were correlated with the electro mechanical cycles In influx experiments (direct monitoring method) however both isotopes exhibited fluctuations in relation to the single heart cycle The interpretation of this observation is discussed The tissue is both structurally and functionally particularly complex the electrical (action potential) and the mechanical (contraction) activities run in parallel and are difficult to dissociate from each other Tracer studies on such a preparation can

not *a priori* be expected to give comprehensible results which reflect the details of ion distribution in the tissues (between the intra and extracellular spaces) The possible effects of mechanical activity are discussed further and it is suggested that an external squeezing effect by the distortion of a critical unstirred surface layer surrounding the preparation may be responsible for some results reported in the literature of an increased tracer efflux associated with the electro mechanical activity

The DMM with which a large number of cycles are analysed makes it possible by a continuous averaging method to calculate a mean cycle This increases the sensitivity of the method enormously *It is even possible to study tracer fluxes so small that they lie within the uncertainty of the analysis method The resolution with respect to time for DMM is relatively good* (this depends on how short the Ringer and silicone oil perfusion periods can be made and is limited for technical reasons) It should be pointed out that DMM always gives tracer flux into the preparation as a whole but as long as internal concentration gradients for the specific activities exist calculations of total flux cannot be made

The mean cycle was calculated for both Br^8 and Na^{24} fluxes There was a maximum for the recorded activity at or immediately after depolarization while the minimum occurred in the diastolic phase The systematic fluctuations may depend either on a correlation between the size of the extra cardiac surface film (O_R) during silicone oil perfusion and the mechanical activity cycles or an electro mechanical effect on the properties of the extracellular space It is concluded that variations in O_R associated with the electro mechanical activity can be excluded as a cause of the oscillations in the mean cycle It is also not clear how extracellular squeezing could bring about such a correlation *If a purely mechanical basis can be excluded the question arises as to whether the results for Na^{24} and Br^8 are biological realities If this is the case it would mean that a Na influx began before depolarization This question and the result for Br^8 for the mean cycle are briefly discussed*

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'UDIES OF CATECHOLAMINE UPTAKE STORAGE AND RELEASE MECHANISMS

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CONTENTS

CHAPTER I

INTRODUCTION

Historical remarks

The first, even if indirect evidence of the presence of catecholamines (CA in the body was obtained by Vulpian (1856), who by a specific colour rea tion demonstrated the occurrence in the adrenal medulla and its venc effluent of chemical substances now known to be identical with CA

Important landmarks in the early development of research in this field we the observations of the marked cardiovascular effects of intravenously i jected adrenal medullary extracts (Oliver and Schafer 1895, Szymor wics 1895 Cybulski 1895) the chemical identification of adrenaline (A (Takamine 1901), the synthesis of both A and noradrenaline (NA) (St 1904) and the proposition that A might function as a chemical transmitter the nerve impulse at the peripheral sympathetic neuro-effector junctio (Elliott 1904)

During the following decades A was unanimously regarded as the excl sive adrenal medullary hormone However, several deviations of the effe of intravenously injected A from those of sympathetic nerve stimulati made the identity of A with the sympathetic neurotransmitter a matter continuous debate The observation that NA more closely reproduced the effects caused several workers to speculate on this amine as a likely ca didate for the neurotransmitter role (Barger and Dale 1911 Bacq 1934 For details concerning this early stage in CA research the reader is refe red to the reviews by Tainter and Ludueña (1950) and Euler (1956)

The demonstration by Euler (1946 a b, 1948) that the sympathetic neur transmitter consists mainly or exclusively of NA may well be regarded as t beginning of a new era in CA physiology At about the same time and i dependently NA was also shown to occur in the adrenal medulla (Hol

[1]) Abbreviations

NA noradrenaline	ATP adenosine triphosphate	
A adrenaline	ADP adenosine diphosphate	
CA catecholamine(s)	AMP adenylic acid	
DA dopamine		
TA tyramine		
NM normetanephrine		

Credner and Kroneberg 1947) both as a precursor of A and as an independent hormone (Hillarp and Hokfelt 1953, 1954, Eranko 1955).

Storage granules

A considerable proportion of the CA in the adrenal medulla (Hillarp, Lagerstedt and Nilson 1953, Blaschko and Welch 1953), in the noradrenergic axones and in sympathetically innervated organs (Euler and Hillarp 1956) is kept in intracellular particles, which can be isolated by differential centrifugation. These storage granules have a very high content of adenine nucleotides (Falck, Hillarp and Hogberg 1956, Blascko et al 1956, Schumann 1958b, Potter and Axelrod 1963b). The remarkable constancy of the molar CA/adenine nucleotide relationship under various conditions has led to the proposition that the CA which are apparently stored in the granules in a non diffusible state, actually form a complex with adenine nucleotides and some third component, probably intragranular protein (Carlsson and Hillarp 1956a, Blaschko, Hagen and Hagen 1957, Hillarp 1958b, Schumann 1958a). However, according to more recent evidence there is a certain deficit in total adenine nucleotides relative to the CA in the adrenal medulla of all species studied, implying the existence of an intragranular pool of CA bound in some different way (Hillarp 1960a, Burack, Draskoczy and Weiner 1961).

Biosynthesis

The last step in the biosynthesis of NA, the β hydroxylation of dopamine (DA) has been shown to occur in the granules which contain the enzyme DA β-oxidase (Kirshner 1957, Potter and Axelrod 1963 b). This implies that the granule fraction functions not only as a storage system, but *is also responsible for the final step in the neurotransmitter synthesis*. The decarboxylation of DOPA (Blaschko, Hagen and Welch 1955) and in chromaffin tissue the methylation of NA (Kirshner and Goodall 1957) take place in the cell sap (Masuoka, Clark and Schott 1958).

Uptake from tissue fluid

While CA are synthesized locally both in noradrenergic nerves and in the adrenal medulla, recent observations clearly indicate that they are also to a

variable extent taken up into tissue stores from the blood stream (Raab and Gigee 1955 Burn and Rand 1958, Muscholl 1960) The existence of a highly efficient mechanism for uptake and retention of circulating CA in the tissues has been elegantly demonstrated by following the fate of intravenously injected radioactively labeled A (Axelrod Weil Malherbe and Tomchick 1959) and NA (Whitby, Axelrod and Weil Malherbe 1961) Chronic postganglionic denervation of sympathetically innervated tissues strongly reduced the uptake of labeled NA (Hertting et al 1961 a) Sympathetic nerve stimulation after previous injection of labeled NA caused release of radioactive neurotransmitter into the blood stream (Hertting and Axelrod 1961)

One may thus conclude that the amines injected into the circulation are specifically concentrated in the noradrenergic axones themselves This is supported by the demonstration with autoradiographic (Wolfe et al 1962) and histochemical (Hamberger et al 1964) methods of exogenous NA accumulating in the axones The specificity of the uptake and retention mechanism is emphasized by the observation that the physiologically occurring L isomer of NA is preferentially taken up and longer retained that the D isomer (Maickel, Beaven and Brodie 1963 Kopin and Bridgers 1963)

In experiments with isolated tissue slices from the cat the amine uptake mechanism has been shown to operate with high efficiency at the low NA levels physiologically occurring in the extracellular fluid (Dengler, Spiegel and Titus 1961 a b, Dengler et al 1962) The accumulation of exogenous NA in the slices proceeded against an apparent concentration gradient and was inhibited by low concentrations of ouabaine or reserpine At high amine concentrations in the medium the amine uptake was no longer prevented by these drugs Similar observations have been made using the isolated perfused rat heart (Iversen 1963) In this case cocaine was shown to strongly inhibit the uptake of NA even at excessively high concentrations of this amine in the perfusion fluid

Comparison of the concentration of labeled NA in isolated tissue preparations and the incubation medium (Dengler et al 1962) as well as studies of the characteristics of the uptake (Iversen 1963) and the multiphasic course of elimination of radioactively labeled NA in isolated perfused organs (Kopin Hertting and Gordon 1962 Axelrod et al 1962) or *in vivo* (Whitby et al 1961 Montanari et al 1963 Udenfriend and Zaltzman Nirenberg 1963) suggest that the NA store in sympathetically innervated tissues is not homogeneous but consists of two or more pools with different turnover rates

Pharmacological effects

The concept of non homogeneity of the NA store in sympathetically inner vated tissues is supported by pharmacological evidence (Trendelenburg 1961, Stjarne 1961, Kopin and Gordon 1962, Weiner, Draskoczy and Bu rack 1962, Crout, Muskus and Trendelenburg 1962, Potter, Axelrod and Kopin 1962, Axelrod et al 1962, Chidsey, Harrison and Braunwald 1962)

Thus the vasoconstrictor response to tyramine (TA) as well as to electrical nerve stimulation, lost after reserpine treatment, could be temporarily restored by an intravenous infusion of CA, which caused no or a barely detec table increase in the tissue content of NA (Burn and Rand 1958, Muscholl 1960 Rosell and Sedvall 1961)

The obvious implication, that TA ows its sympathomimetic effects to its capacity to release NA from sympathetically innervated tissues (Fleckenstein 1953) has been confirmed (Lockett and Eakins 1960, Burn and Burn 1961, Lindmar and Muscholl 1961, Stjarne 1961, Axelrod et al 1962, Chid sey et al 1962), while it has also been established that the adrenal medulla does not contribute to the cardiovascular effects of TA (Stromblad 1960, Stjarne 1961, Weiner et al 1962a)

This is surprising in view of the fact that TA has been shown to release CA from isolated storage granules both from the adrenal medulla (Schu mann and Weigmann 1960) and from noradrenergic axones (Euler and Lishajko 1960 Schumann and Weigmann 1960) on incubation *in vitro*

The apparent discrepancy between the *in vitro* and *in vivo* effects of TA led to the proposal that TA may exert its NA releasing effects *in vivo* at a level different from that of the specific storage granules, possibly on a NA pool bound to the axonal membrane (Stjarne 1961, Weiner et al 1962 a)

The fact that the sympathomimetic effect of TA, lost after reserpine treatment, could be temporarily restored by a NA infusion, leading to a barely detectable increase of the NA content of the organs, indicates that the TA sensitive pool may be quite small (Burn and Rand 1960 Crout et al 1962)

TA released labeled NA, previously taken up into the heart, both from the isolated perfused organ (Axelrod et al 1962) and *in vivo* (Chidsey and Harrison 1963 Potter and Axelrod 1963 a) The specific activity of the released amines was found to vary depending on the time interval after the labeled amine was administered (Potter et al 1962 Chidsey and Harri son 1963) At the time when complete tachyphylaxis to the cardioacceler ating action of TA had developed the CA content of the heart was only

moderately reduced (Axelrod et al 1962) and the response to nerve stimulation was normal (Cowan et al 1961, Harrison Chidsey and Braunwald 1963)

Thus the results obtained with TA strongly support the concept of non homogeneity of the NA store of sympathetically innervated tissues

Another drug which has proved to be a highly efficient pharmacological tool for analysis of the physiology of the CA is reserpine For the details of the vast literature the reader is referred to the reviews by Shore (1962) and Carlsson (1964)

Reserpine administration causes a profound depletion of the NA stores of peripheral noradrenergic nerves leading to failure of the impulse transmission at the sympathetic neuro-effector junction and also to abolishment of the characteristic cardiovascular effects of intravenous TA injection (Bertler Carlsson and Rosengren 1956, Carlsson and Hillarp 1956b, Holzbauer and Vogt 1956) In some animal species and organs the full depleting effect of reserpine is exerted only in the presence of intact innervation (Holzbauer and Vogt 1956 Kroneberg and Schumann 1957 Carlsson et al. 1957a Frankö and Hopsu 1958 Karki Paasonen and Vanhakartano 1959 Stjärne and Schapiro 1959, Hillarp 1960b Mirkin 1961, Benmiloud and Euler 1963) Thus unilateral sectioning of the sympathetic chain immediately prior to reserpine administration resulted in the preservation of about 20 per cent of the original amount of NA in skeletal muscle in the cat on the denervated side at a time when the innervated side was completely depleted The NA remaining proved to be resistant to further reserpine injections while it was readily released by electrical nerve stimulation producing vasoconstriction (Sedvall and Thorsson 1963) Since the available data do not demonstrate an increased nerve impulse traffic in the peripheral sympathetic nerves after reserpine administration (Iggo and Vogt 1960) these findings suggest that intermittent depolarization may play a permissive role in the mechanism of reserpine-induced depletion of the CA stores (Hertting Potter and Axelrod 1962) Moreover, they suggest the existence of a small axonal pool of NA sequestered from the rest of the intraaxonal NA and immediately related to impulse transmission at the noradrenergic neuroeffector junction

This is supported by the observation that NA administration leading to accumulation of only 1—2 per cent of the normal NA content was enough to produce temporarily a nearly complete restoration of the function lost due to reserpine induced depletion of the NA stores (Crout et al 1962) Apparently only a minor part of the NA normally present in the axone is at any given moment immediately concerned with impulse transmission

The mechanism of the depletion of the CA stores caused by reserpine is as yet poorly understood However, the *in vitro* evidence indicates that the reserpine concentrations, which may be expected to occur in the tissues, at least with the lowest *in vivo* dose which has a full depleting action, have no direct amine releasing effects on the storage granules from bovine noradrenergic nerves (Euler and Lishajko 1961c), from the rat heart (Potter and Axelrod 1963b) or from bovine or rabbit adrenal medulla (Stjarne and Schapiro 1959, Euler and Lishajko 1961c, Weil Malherbe and Posner 1963) Moreover, the rapid and nearly complete depletion of most sympathetically innervated tissues induced by reserpine is not accompanied by a corresponding flooding of the extracellular fluid with intact, biologically active NA (Carlsson et al 1957a, Burger 1957, Bertler et al 1958) but rather by the appearance of large amounts of deaminated metabolites (Kopin and Gordon 1962, 1963a), indicating that the majority of the amines released by reserpine is enzymatically inactivated prior to departure from the axone (Carlsson et al 1957a, Kopin and Gordon 1962, Carlsson 1964)

Decreased provision of NA to the stores by depressed local synthesis and inhibited uptake from the extraaxonal fluid appears to be a factor of importance for the development and prolonged maintenance of amine depletion after reserpine treatment The capacity of the axonal stores to take up and/ or retain circulating CA is strongly reduced by reserpine (Muscholl 1960 Hertting Axelrod and Whitby 1961, Kopin et al 1962, Axelrod et al 1962 Andén Carlsson and Waldeck 1963 Andén Magnusson and Waldeck 1964) The similar inhibition of NA uptake in isolated tissue slices has been interpreted as a reserpine induced block of the specific amine transport mechanism of the axonal membrane (Dengler et al 1962) The specific ATP Mg dependent mechanism for uptake of CA including DA into the synthesis and storage granules, both from noradrenergic nerves (Euler and Lishajko 1963b) and adrenal medulla (Carlsson Hillarp and Waldeck 1962 1963 Kirshner 1962, Kirshner Rone and Kamin 1963) is also strongly inhibited by reserpine Insufficient synthesis due to a block of the uptake of DA and subsequent failure to replenish the stores of CA lost through physiological release have been regarded as the main factors responsible for the amine depletion caused by reserpine (Bertler 1961 Kirshner 1962)

However at low concentrations reserpine also strongly inhibits the spontaneously occurring outflow of amines from the storage granules into the external medium on incubation *in vitro* both in nerve (Euler and Lishajko 1961c and adrenal medullary granules (Euler and Lishajko 1962

Weil Malherbe and Posner 1963), which would tend to counteract depletion

Evidently reserpine is able to interfere with specific CA transport at more than one level, counteracting both in and outward passage of amines at the level of the storage granules, and possibly in a similar way affecting amine transport across the axonal membrane

Fate of the ATP lost on amine secretion

The disappearance of ATP from the adrenal medulla on stimulation of amine secretion *in vivo* (Carlsson and Hillarp 1956a, Carlsson Hillarp and Hokfelt 1957, Schumann 1958a Burack, Weiner and Hagen 1960) was not accompanied by accumulation in the gland of any if its possible breakdown products neither ADP, AMP, adenosine or other compounds having an ultraviolet absorption spectrum characteristic of the adenine nucleus nor of inorganic phosphate Thus it was concluded that "both catechol amines and ATP (or its split products) leave the cells rather promptly after being released from the granules (Carlsson et al 1957b)

The CA released from isolated adrenal medullary granules on incubation in vitro were recovered as intact amines in the incubation medium (Weil Malherbe and Posner 1963), while the ATP lost from the granules was quantitatively transformed to AMP (Hillarp 1958d) However, results obtained with the hexokinase trap technique showed that at least 50 per cent of the ATP disappearing from the granules left them unchanged without having been dephosphorylated This led to the conclusion that "it may not seem so unlikely as previously thought that ATP may be secreted from the medulla along with the sympathomimetic amines (Hillarp 1958d)

The present investigation

The present investigation deals with problems concerning the uptake, storage and release of CA, and with the modification of these processes induced by drugs notably reserpine It is divided into three sections

In the first the purpose was to analyse the quantitative and possible qualitative differences between isolated granules from bovine noradrenergic nerves and adrenal medulla particularly with regard to amine and ATP storage mechanisms homogeneity of the pools of amines stored in

these particles, turnover rates of the amines and in the effects of certain drugs, including reserpine

In the second section the relevance of some of the results obtained *in vitro* was assessed by *in vivo* experiments Thus the time course of the uptake and elimination of intravenously injected tritiated DL-NA in the rabbit heart as a whole, and in the specific granule fraction isolated from the heart by differential centrifugation, was studied The mechanism of action of reserpine was analysed by following the time course of the NA depletion induced by this drug, as well as that of the simultaneously appearing inhibition of the uptake of circulating NA, in the rabbit heart

In the third section the fate of the ATP lost from the adrenal medulla, concomitantly with CA secretion, was studied by direct analysis of the effluent from the perfused bovine adrenal gland In addition an attempt was made to investigate the biosynthesis pathway and turnover rate of the ATP in the adrenal medulla by analysing the incorporation of radioactively labelled adenine into the various purine and nucleotide fractions of the gland Finally, changes in the ribonucleic acid fraction of the adrenal medulla, accompanying the CA and ATP loss during perfusion, were studied by chromatographic analysis of the bases obtained by hydrolysis of the acid insoluble material in the medulla

CHAPTER II

EXPERIMENTS WITH ISOLATED GRANULES

Introduction

While the CA content of the adrenal medullary granules is so high that enough material for *in vitro* experiments can easily be obtained even from smaller laboratory animals the study of the nerve granules has required the use of large amounts of noradrenergic nerve tissue, which for technical reasons has led to the routine use of bovine splenic nerves for this purpose. Using the technique of labeling the NA of the tissue stores by pretreatment with tritiated NA similar studies could recently be carried out in granules isolated from the rat heart (Potter and Axelrod 1963b)

On isolation of the storage granules of the adrenal medulla by differential centrifugation 70—80 per cent of the total amount of amines were found in the granules (Blaschko and Welch 1953 Hillarp et al 1953, Schumann 1958a, Hillarp 1960a), while the corresponding fraction in nerve tissue contained 25—30 per cent of the amines (Euler 1958, Schumann 1958b)

Morphology

The morphological characteristics of the granules have been studied by electron microscopy, either *in situ* or after isolation The size of the medullary granules has been reported to range from 500 Å to 6000 Å in different species (Lever 1955 Sjostrand and Wetzstein 1956 De Robertis and Ferreira 1957 Lever, Lewis and Boyd 1959, Hagen and Barrnett 1960 Hillarp 1960c Kleinschmidt and Schumann 1961, Wetzstein 1961) The nerve granules are stated to be smaller Thus the size of the granules isolated from bovine splenic nerves ranged from 600 Å to 2000 Å (Euler 1961) In the vas deferens of the rat the size of the granules ranged from 300 Å to 900 Å (Richardson 1962) The granules are surrounded by a well defined membrane the thickness of which is reported to be 30—100 Å in medullary granules The central core of the granules contains smaller particles of a size of 175—200 Å which may represent fixation artefacts but

could on the other hand also be regarded as morphological evidence of a structural organization of the stroma of the granules (Wetzstein 1961)

Chemical composition

By gradient centrifugation techniques it was possible to obtain a preparation of bovine medullary granules which was practically free of contaminating mitochondria or microsomes (Hillarp 1958c) The water content of these granules was 68 5 per cent CA made up 20 per cent, adenine nucleotides 15 per cent, protein 35 per cent and lipids 22 per cent of the dry weight (Hillarp 1959) CA and adenine nucleotides represented practically all of the cationic and anionic constituents of the granules, respectively (Carlsson and Hillarp 1958) The membrane of the granules was reported to be permeable to Na , K , sucrose and CA

While the chemical characteristics of the medullary granules have thus been extensively studied, corresponding data for the nerve granules are not available

Adenine nucleotides

The adenine nucleotide content of the adrenal medulla of all species studied has been shown to be very high In bovine medulla, which contained some 10 μmoles of ATP per gram wet weight (Falck et al 1956) ATP was the dominating nucleotide representing 77 per cent of the total adenine nucleotides while ADP and AMP made up 14 and 9 per cent, respectively (Hillarp 1960a) In other species ATP is less dominating Thus the relative proportions of ATP, ADP and AMP in the rat medulla were 68, 25, and 7 per cent (Hillarp Jonsson and Thieme 1959) and in the fowl 59, 24 and 17 per cent (Burack et al 1960)

The quantitative relationship of the CA to the adenine nucleotides in the medulla shows a remarkable constancy The positive charges of the CA were balanced by the sum of the negative charges of the different adenine nucleotides (Falck et al 1956 Blaschko et al 1956 Schumann 1958a) When ATP is the only nucleotide a CA/ATP ratio of 4/1 gives an "equivalence ratio of unity In other cases the sum of the different adenine nucleotides had to be used to obtain "equivalence with the amines (Hillarp and Thieme 1959 Burack et al 1960)

The equivalence ratio was remarkably well maintained both in whole

medulla and in isolated granules under a wide variety of conditions of amine depletion and repletion (Carlsson and Hillarp 1956a Carlsson et al 1957b, Hillarp 1958d, 1960a, Schumann 1958a, Weiner, Burack, and Hagen 1960) However, there are some reports of marked changes of this ratio on amine depletion induced by reserpine and insulin (Schumann 1958a Burack et al 1960), indicating that under some circumstances the amines and nucleotides may vary independently of each other

The adenine nucleotides of the adrenal medulla occur almost exclusively in the amine storage granules and show a remarkable stability, not being broken down to any appreciable extent during the process of isolation of the granules (Falck et al 1956) The ATP of the medullary granules also differs from that of mitochondria in having a very much slower turnover rate, as judged by the incorporation of radioactive phosphorus (Prusoff et al 1961)

The nerve granules (Schumann 1958b Euler, Lishajko and Stjarne 1963) as well as the particulate amine containing fraction isolated from the rat heart (Potter and Axelrod 1963b) have also been shown to contain ATP in amounts giving a CA/ATP relationship close to an equivalence ratio of unity

Amine storage mechanism in the granules

As mentioned above the CA and adenine nucleotides make up almost all of the ionic constituents of the "pure bovine medullary granules occurring at concentrations calculated to be 0 55 M and 0 13 M, respectively (Hillarp 1959) If they existed as free ions the storage granules would contain solutes at twice the osmolarity of blood Thus the amines and nucleotides must be assumed to occur in the granules in an osmotically inactive form In favour of this is also the finding that radioactively labeled A although apparently being able to penetrate into the granule water at 0°C *in vitro* did not exchange to any appreciable extent with the endogenous amines at this temperature (Hillarp 1959) The CA stored in the granules have therefore been assumed to exist in an osmotically inactive non diffusible state forming a complex with adenine nucleotides and some third intragranular component, probably soluble protein, which accounts for up to 80 per cent of the total intragranular protein (Hillarp 1958d)

The CA in the adrenal medulla of several species have recently been reported to exist in excess of the total adenine nucleotides relative to an equivalence ratio of unity implying that more than 20 per cent of the

amines are stored independently of nucleotides This applied to the isolated storage granules as well indicating the probable existence of two different granule bound pools of CA, with different binding mechanisms (Hillarp 1960a Burack et al 1961)

Stability

Bovine medullary and nerve granules are quite stable at 0°C and at physiological pH in various isotonic media, including sucrose (Hillarp and Nilson 1954), potassium phosphate, sodium and potassium chloride (Euler and Lishajko 1961a)

However, on freezing and thawing they behave differently This treatment caused about 75 per cent release of the amines from medullary granules (Hillarp and Nilson 1954) while less than 25 per cent of the NA was released from the nerve granules (Euler and Lishajko 1961a)

Similarly, the medullary granules are highly sensitive to a rapid lowering of the tonicity of the medium, while the nerve granules are relatively resistant A sudden lowering of the tonicity from 0 8 M to 0 5 M sucrose caused a rapid 50 per cent depletion of the medullary granules while reduction to 0 25 M, i e isotonicity resulted in complete depletion Thus the relative, rather than the absolute magnitude of the tonicity change appears to be the decisive factor (Hillarp and Nilson 1954) Osmotic lysis by exposure to distilled water has in fact become accepted as a means of extraction of the medullary granules (Kirshner 1962) In contrast the nerve granules are rather resistant to osmotic lysis Exposure to distilled water for 35 minutes at +5°C caused at the most a 40—50 per cent depletion Euler and Lishajko 1961a)

Both the medullary and the nerve granules are quite resistant to minor variations of the pH of the medium However, at pH 5 and +3°C the medullary granules lost 50 per cent of their CA during 60 minutes while at pH 4.5 the depletion was complete (Hillarp and Nilson 1954) In the nerve granules a 50 per cent depletion was obtained at pH 4 during 10 minutes at 20 C and a complete depletion at pH 3—3 5 (Euler and Lishajko 1961a

Spontaneous release on incubation in vitro

Incubation in various isotonic media at pH 7 5 led to a spontaneous and parallel temperature dependent release of CA and adenine nucleotides

from the medullary granules. At 37°C about 20 per cent were released during the first and some additional 10 per cent during the second hour (Hillarp 1958d).

The release of CA from the nerve granules occurs at a much higher rate. Thus on incubation at 37°C these granules as well as those isolated from the rat heart (Potter and Axelrod 1963b) lost about 50 per cent of their CA in less than 10 minutes (Schumann and Weigmann 1960, Euler and Lishajko 1961a). In the nerve granules the ATP loss was not parallel, but occurred much more slowly (Euler et al. 1963a). Because of the high spontaneous rate of CA release at 37°C many studies of nerve granule physiology have been carried out at a lower temperature.

Prevention of spontaneous loss of CA

It is possible to counteract the spontaneously occurring depletion of the nerve granules on incubation *in vitro* in several ways.

Thus addition of NA to the external medium at a concentration of 1.8×10^{-5} M considerably delayed while 6×10 M completely prevented the net decrease of the NA content of the nerve granules on incubation at 20°C for 60 minutes (Euler and Lishajko 1961d). This "protective" effect is not specific for NA. Addition of A was equally effective in maintaining the original total CA content of the granules (Euler and Lishajko 1962), the uptake of A approximately corresponding to the release of NA. Thus the mechanism of the "protective" action of CA addition to the medium consists in establishment of an inward flux of amines, which will partly or wholly balance the spontaneously occurring outward flux (Euler and Lishajko 1963a).

Similarly addition of ATP or ADP at concentrations of 1—3×10^{-3} M in the presence of Mg strongly counteracted the tendency to spontaneous decrease of CA in the nerve granules (Euler and Lishajko 1963c).

Reserpine added to the incubation medium at concentrations of 10^{-8} M —10^{-5} M (Euler and Lishajko 1961c) the vasodilating agent Segontin (N (3 phenylpropyl (2)) 1,1 diphenylpropyl (3) amine) at 3×10^{-6} M —3×10^{-4} M and the *a* receptor blocking agent phenoxybenzamine at 2.5×10^{-5} M—4×10^{-4} M (Euler, Stjärne and Lishajko 1964) also more or less completely prevented the spontaneous fall in nerve granule NA on incubation *in vitro*.

In bovine adrenal medullary granules the tendency to spontaneous decrease of the CA on incubation is so small that studies of inhibitory mechanisms are difficult. However in isolated medullary granules from the

rabbit, which have a higher rate of spontaneous release of CA on incubation *in vitro*, reserpine has been shown to have a 'protective' effect on the CA similar to that found on nerve granule CA (Euler and Lishajko 1962, Weil-Malherbe and Posner 1963).

Granule uptake of exogenous amines

While CA added to the incubation medium at 0°C are reported to penetrate the bovine adrenal medullary granule membrane without exchanging with the intragranular, endogenous, amines to any measureable extent (Carlsson and Hillarp 1958, Hillarp 1959), a definite exchange did occur at higher temperature (Carlsson and Hillarp 1961). The uptake of exogenous amines was strongly accelerated by addition of ATP-Mg to the incubation medium (Carlsson et al. 1962, 1963a, Kirshner 1962). The uptake mechanism appears to have a limited specificity, since DA, NA and A were taken up to about the same extent, while 5-hydroxytryptamine was taken up even more avidly. A similar ATP-Mg stimulated mechanism for uptage of exogenous CA has been demonstrated in nerve granules (Euler and Lishajko 1963d).

The specific accumulation of exogenous amines was inhibited by many different agents both in medullary (Carlsson et al. 1962, 1963a, Kirshner 1962) and nerve granules (Euler, Stjärne and Lishajko 1963). Thus reserpine at 10[illegible] M to 10[illegible] M caused a 90—95 per cent inhibition of the uptake. Similar effects were found in medullary granules with Segontin 6×10[illegible] M—6×10[illegible] M and with phenoxybenzamine 3.3×10[illegible] M—8×10[illegible] M, and also to a variable extent with several other drugs.

The present investigation

As mentioned previously the object of the present experiments was to compare the storage granules from nerve and adrenal medulla under a series of different *in vitro* conditions and to deliberately emphasize the quantitative and possible qualitative differences in the response of the granules from the two sources.

Methods

Preparation

Bovine splenic nerves and adrenal medulla were obtained at the slaughter house 20—60 minutes after the animals were killed and immediately chil

led with ice All steps from this point to the beginning of the incubation and during the extraction were carried out at ice water temperature

After carefully dissecting the nerve trunks and the medulla free of contaminating tissue they were squeezed between nylon rollers according to the method of Euler (1958) the press juice being diluted with 0 13 M potassium phosphate buffer pH 7 5 to a concentration of 100—200 mg nerve tissue per ml and 10 mg medullary tissue per ml respectively Coarser tissue particles were removed by centrifugation at 600—1000×g for 10 minutes and the supernatant fine granule suspension was used for the incubations

Incubations

Incubations were carried out in stoppered polypropylene or cellulose nitrate centrifuge tubes at 37°C without shaking Control tubes were kept in ice water

Extraction

After the incubation period the tubes were centrifuged at 30000×*g* (medullary granules) or 50000×*g* (nerve granules) for 30 minutes The supernatant was decanted and in some experiments kept for amine and radioactivity assay In experiments with addition of tracer material, the sediment was resuspended in 9—10 ml of phosphate buffer and recentrifuged at 30000—50000×*g* for 30 minutes in order to remove non bound radioactive material dissolved in the granule pellet water After decanting this second supernatant which was also saved for assay of radioactivity the sediment was extracted with 0 5 ml of 0 4 M perchloric acid The sediment was carefully resuspended in the extraction medium and kept in this for 5—15 minutes After dilution with glass-distilled water the tubes were recentrifuged at 30000—50000×*g* for 10 minutes The clear supernatant was decanted 1 ml potassium phosphate was added the extract brought to pH 6 5 with 0 4—1 M potassium hydroxide and immediately assayed for CA ATP and in some experiments radioactivity

CA assay

CA were assayed fluorimetrically according to the method of Euler and Lishajko (1961b) The recovery of added NA and the accuracy of the determinations were found to be satisfactory (Table I) The presence of adenine nucleotides did not interfere with the CA assay

Table 1 *To eight test tubes containing equal aliquots of nerve granule sediments extracted with 0 5 ml ice cold 0 4 M perchloric acid were added different amounts of ATP and NA Further treatment of the extracts according to method described*

TUBE	ADD		NA FOUND		NA EXP	RECOV	ATP FOUND		ATP EXP	RECOV
No	NA µg	ATP nano-moles	µg	Mean of dupl	µg	per cent	nano-moles	Mean of dupl	nano moles	per cent
1	—	—	2 04	2 09	—	—	2 91	2 83	—	
2	—	—	2 13				2 75			
3	0 5	1 0	2 57	2 56	2 59	98	3 70	3 67	3 83	96
4	0 5	1 0	2 55				3 64			
5	1 0	2 0	3 06	3 05	3 09	99	4 55	4 38	4 83	91
6	1 0	2 0	3 04				4 20			
7	1 5	3 0	3 51	3 54	3 59	99	5 85	5 85	5 83	100
8	1 5	3 0	3 57				5 85			

ATP assay

ATP was determined by a modification of the luciferase method of Strehler and Totter (1954)

Dried firefly lanterns were ground in a porcelain mortar and extracted with ice cold 0 1 M sodium arsenate buffer pH 8 0 After centrifugation at 3000 × g for 10 minutes in the cold the supernatant was decanted and diluted with buffer to a final concentration of 0 25 mg of firefly lantern per ml The activity of the luciferase preparation decreased with time the slope of the decay curve being rather steep during the first few hours but later levelling out It was therefore made up several hours before use and kept in ice water

Fresh ATP standard was prepared each day and diluted with ice cold glass-distilled water to 10^{-6} M

The assay was carried out using a photomultiplier tube (E M I 6255 A) operated at 1775 volts The signal was fed into a Speedomax Type G recorder

The light producing reaction took place in ordinary glass Beckman

cuvettes (10 mm) Six cuvettes contained in a holder were placed in a light tight box as closely as possible to the photo multiplier tube A hypodermic needle projected through the cover of the box to a point just above the cuvette in position, so that mixing was rapid when the reaction was initiated by injection of the luciferase solution

The luciferase reaction is affected by high salt concentration (Cheng 1961) To create uniform conditions for the ATP standard and the extract a constant extract volume of 0 5 ml was always used and the same volume of solvent (perchloric acid/potassium phosphate buffer/potassium hydroxide) was added to each standard ATP cuvette

The procedure was carried out as follows 0 2 ml 0 1 M $MgSO_4$ 0 5 ml extract (ice cold) and 1 8 ml glass distilled water (for ATP standard cuvettes water+0 5 ml solvent) were added to the cuvettes at room temperature After transferring them to the light tight box a constant amount of ice cold luciferase solution (varying according to the enzyme activity level from 0 5 to 1 ml) was injected into the cuvettes The decrease with time of the activity of the firefly extract made it necessary to include ATP standards in each run

The relationship between the amount of ATP in the samples and the deflection of the recorder was not always linear However the accuracy of the method was checked by recovery experiments and was found to be satisfactory (Table I) The luciferase reaction was not absolutely specific for ATP ADP was about one tenth as effective as ATP and also caused a light emission rising to its maximum much more slowly Thus the presence of about 10 per cent ADP contaminating the samples did not appreciably affect the accuracy of the ATP determinations CA did not disturb the luciferase reaction

Radioactivity determination

Radioactivity was measured in a Packard Tri Carb Liquid Scintillation Spectrometer Aqueous samples were counted in a 7 3 toluene absolute ethanol solution containing 4 g of 2 5 diphenyloxazole and 100 mg of 1 4 bis 2(5 phenyloxazolyl) benzene per liter of toluene Monitoring by internal standards showed no difference in quenching activity between the samples in each experimental series Most samples were run in duplicates showing good agreement The counting time was generally 10 minutes

Radiochemical impurities in the commercial H^3DL-NA preparation used were removed and the identity of the radioactive compounds found in

tissue extracts was checked by ion exchange chromatography using Amberlite CG 120, Type 2 (200—400 mesh) according to Haggendal (1963)

Substances used

L-NA bitartrate (Byk Gulden)
D NA bitartrate (Sterling Winthrop)[1]
DL-NA base (Schuchardt)
DL-NA 7-H^3 hydrochloride (New England Nuclear Corp) Specific activity 7 06—7 60 C/mM
Adenosine 5' triphosphate, disodium salt (Sigma)
Dried firefly lanterns (Sigma)
Reserpine phosphate, lyophilized (Ciba)
Segontin gluconate (Hoechst)
Phenoxybenzamine (Smith Kline and French Lab)

Presentation of data

The results are expressed on a molar basis throughout

[1] Kindly put at our disposal by Dr F P Luduena Sterling Winthrop
Kindly put at our disposal by Dr A J Plummer CIBA Summit N J

Results and discussion

1 CA AND ATP CONTENT OF NERVE AND MEDULLARY GRANULES

Results

The CA and ATP content and CA ATP ratio of isolated bovine splenic nerve and adrenal medullary granules are presented in Table II

Table II *CA and ATP content and CA/ATP ratio of isolated bovine splenic nerve and adr nal m dullary granul s CA and ATP in the nerve granules are expressed in nan mo es pe gram ner and n the medullary granules in μmoles per gram medulla (wet weights Mean values of 10 experiments ± standard error of mean*

	NA	A	NA+A	NA %	ATP	CA/ATP
Nerve granules	[illegible]				3 4? ± 0 [illegible]	4 39 ± 0 60
Medullary granules	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	5 57 ± 0 ?0

Discussion

In the nerve granules the values found for NA and ATP were higher than those reported by Schumann (1958b) but the CA/ATP ratio was of the same order of magnitude as that found by him The specific amine storing particulate fraction from the rat heart was reported to have the same relative NA and ATP content (Potter and Axelrod 1963b) Because of the small amount of nucleotides present in the nerve granule extracts it has not yet been possible to analyse them by anion exchange chromatography for ADP and AMP Even with a small contribution by such adenine nucleotides an equivalence ratio of unity might well be obtained The present findings are thus compatible with the concept of CA storage in the nerve granules by complex formation with adenine nucleotides and some intragranular protein (Hillarp 1958b)

However there are reasons to assume that the nerve granule sediment is not free of contamination by other intraaxonal particles which may also contain adenine nucleotides In that case the true equivalence ratio for the nerve granules would be considerably higher than unity implying that at least part of the amines in the nerve granules must be stored in a different way by a mechanism not dependent on a stoichiometric CA/adenine nucleotide relationship Such a storage mechanism obviously exists in chromaffin tumour granules where only a minority of the amines could possibly be stored together with equivalent amounts of adenine nucleotides (Gelinas Pellerin and D Iorio 1957, Schumann 1960, Stjarne Euler and Lishajko 1964)

The CA and ATP content and the molar ratio CA/ATP of the medullary granules were in the same range as those found in washed "crude bovine adrenal medullary granules by Falck et al (1956) The molar CA/ATP ratio corresponded to an equivalence ratio of 1 38 Even including ADP and AMP in the proportions mentioned above to be characteristic of bovine adrenal medullary granules 14 and 9 per cent of the total adenine nucleotides respectively, the equivalence ratio would not be less than 1 20 indicating an excess of CA over the total amount of adenine nucleotides available Thus part of the amines of the medullary granules apparently also has a storage mechanism not requiring equivalence of amines and adenine nucleotides

2 EFFECTS OF CHANGES IN TONICITY ON CA AND ATP IN THE GRANULES

Methods

A special method was devised for a study of the time course of the changes induced in the CA and ATP content of the granules on exposure to hypotonic media

The experiments were carried out at 0°C To a 5 ml aliquot of a suspension of nerve or medullary granules in 0 13 M potassium phosphate buffer, pH 8 2, contained in an ice cooled glass beaker, were added 1 g of aluminium oxide and in some experiments 1 g of the strong anion exchange resin Dowex 2-X8 (200—400 mesh formate form) The suspension was kept well mixed with a magnetic stirrer In this way CA or adenine nucleotides released from the granules were continuously removed by adsorption on the aluminium oxide and the resin, respectively By addition of ice cold glass-distilled water the tonicity of the medium was rapidly changed After different time intervals the suspension was forced by compressed air through a glass column containing aluminium oxide (10 × 20 mm) The passage through the column was accomplished in 10—20 seconds The effluent was collected in a glass beaker containing enough of 4 M perchloric acid to give a final concentration of 0 4 M From this point onwards the perchloric acid extract was treated as described earlier (see under Methods) and analysed for CA and ATP

The CA left on the column, representing amines released from the granules during the experiment were eluted according to the standard method and analysed fluorimetrically as a check of the method

Nerve granule suspensions have previously been shown to pass unimpaired through aluminium oxide columns Euler and Lishajko 1961a, Direct measurement of the CA released from the granules during the experiment and thus adsorbed on the aluminium oxide and calculation of the release by determination of the amine deficit in the granule suspension after passage through the column showed good agreement

The presence of the ion exchange resin in the medium proved to induce a partial and rapidly completed loss of amines and nucleotides from the medullary granules even in isotonic media see Fig 2) Thus the results obtained in the experiments where resin was added represent the additional effect induced by hypotonicity The depletion caused by the resin alone was even more marked in the nerve granules No study of the effect of shift in the osmotic pressure on the ATP in these granules could be carried out

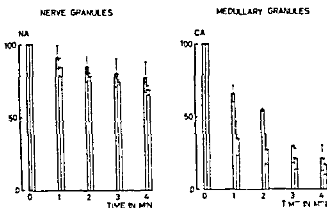


Fig 1 Effect of hypotonicity on the CA content of isolated nerve and adrenal medullary granules on incubation at 0 C in the presence of aluminium oxide
Ordinates CA remaining in granules (per cent of starting values)
Left columns osmolarity reduced to 1/3
Right columns Osmolarity reduced to 1/6
Means and range (2 experiments)

Results

The nerve granules proved to be much more resistant than the medullary granules to exposure to hypotonic media (Fig 1) Thus a lowering of the tonicity of the medium to 1/3 for 4 minutes caused a fall in CA in the nerve granules of 23 per cent and in the medullary granules of 79 per cent while at a tonicity of 1/6 the nerve granules lost 31 per cent and the medullary granules 83 per cent of their CA

The fall in medullary granule CA induced by hypotonicity was not paralleled by the decrease in ATP, which was almost completely eliminated from the granules in less than 1 minute by lowering the osmotic pressure to 1/2 or less (Fig 2)

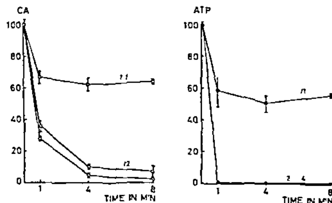


Fig 2 Effect of hypotonicity on the CA and ATP content of isolated adrenal medullary granules on incubation at 0 C in the presence of aluminium oxide and anion exchange resin (Dowex 2) Osmotic pressure reduced to 1/2 and 1/4 respectively Controls incubated at isotonicity
Ordinates CA and ATP remaining in the granules (per cent of starting values)
Left CA Right ATP
Means and range (2 experiments)

Discussion

The present findings firstly emphasize that the nerve granules are far less sensitive to changes in the tonicity of the medium than are medullary granules This is in agreement with previous observations in this laboratory (Euler and Lishajko 1961a)

They also give some hints as to the state of the amines in the granules The fairly rapid initial phase of the amine release from the nerve granules indicates that the sudden lowering of the osmotic pressure in the medium induced structural changes in the granules which allowed water soluble intragranular amines to diffuse across the granule membrane into the external medium However the slowness of the continued release suggests that the majority of the amines in these granules are not dissolved in the intragranular water neither as free ions, nor in any form of water soluble complex but rather exist in relatively firm binding to the stroma

In the medullary granules the sudden lowering of the tonicity of the medium caused a rapid initial outflux of amines leading to a partial depletion After the initial rapid release phase which was largely completed in less than one minute the amount of intragranular amines tended to stabilize at the new level This is in agreement with the findings of Hillarp and Nilson (1954) who by direct measurement demonstrated that the new amine level resulting from the initial release was largely established in less 20 minutes and that this level was then maintained for up to 24 hours By studying the optical changes accompanying the CA release on exposure to hypotonic media they were able to conclude that the structural changes underlying the osmotically induced amine relase were irreversible and completely developed in less than 2 5 minutes

On exposure to hypotonicity the ATP vanished much more rapidly from the medullary granules than did the CA Whereas the ATP was almost completely eliminated the CA level of the granules tended to stabilize at a lower level

The apparent independence of the variations in CA and ATP may imply that they occur in the granules independently and that the maintenance of nearly equivalent amounts of intragranular amines and nucleotides is just coincidence Alternatively one may conclude as proposed by Hillarp 1960a that part of the CA of the adrenal medullary granules is stored in a complex with an equivalent amount of adenine nucleotides and protein while another part of the amines is stored in a different way possibly independent of adenine nucleotides According to Hillarp 1960a)

the non nucleotide bound CA pool may represent more than 20 per cent of the total granule bound amine population

If the second alternative is correct one may conclude from the present experiments that hypotonicity induces structural changes in the medullary granules which cause a rapid and nearly complete, selective release of the nucleotide protein bound CA pool, while amines bound in some different way possibly to the stroma remain for some time in the lysed granules

Different proportions of these two fractions in storage granules from noradrenergic axones, adrenal medulla and chromaffin tumours may thus be the basis of the characteristic differences in the relative amounts of CA and adenine nucleotides in such storage granules

The following arguments seem to support the proposition of stroma binding as the mechanism of storage of part of the intragranular CA First ly electron microscopy has revealed an intragranular pattern suggesting the possible existence of subgranules (Sjostrand and Wetzstein 1956 Wetzstein 1961), one obvious feature of which would be to provide surface enlargement Secondly, the adrenal medullary granules are rich in lipids, which make up 22 per cent of their dry weight (Hillarp 1959) Thirdly, certain lipids are known to have a high pH-dependent binding capacity for CA (Euler 1946a Norlander 1950) In fact very large amounts of NA have been recovered from the lipid phase of a chromaffin tumour extract (Euler 1956)

Lipids are known to be concentrated in oriented layers at biological membranes It is conceivable that lipids may occur at the surface limiting structures in the interior of the granules from both noradrenergic axones adrenal medulla and chromaffin tumours Thus binding to lipids or lipoprotein in the granule stroma may possibly be the mechanism whereby high concentrations of non adenine nucleotide bound CA are maintained in the storage granules

3 RELEASE OF CA AND ATP ON INCUBATION IN VITRO

Results

Incubation at 37°C for varying times caused a relatively small loss of CA and ATP from the medullary granules There was thus only a moderate shift in the molar CA/ATP ratio from 5 85 (range 5 76—5 95) to 5 15 (range 4 90—5 50)

In the nerve granules under the same circumstances the NA was rapidly lost a 50 per cent reduction being obtained in 10 minutes The ATP decreased much more slowly (Fig 3), leading to a drastic reduction in the molar CA/ATP ratio from 4 75 (range 3 65—5 36) to 0 82 (range 0 75—0 87)

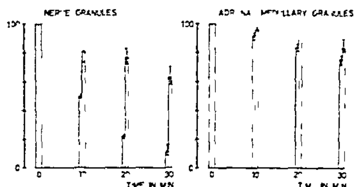


Fig 3 Decrease in CA and ATP in nerve and adrenal medullary granules on incubation at 37 C
Ordinates CA and ATP remaining in the granules (per cent of starting levels)
Left columns CA
Right columns ATP
Means and range (3 experiments)

Discussion

The results of the incubation experiments clearly demonstrate the well known difference in stability of the CA of the storage granules from bovine noradrenergic axones and from adrenal medulla on incubation at 37°C Hillarp 1958d Schumann and Weigmann 1960 Euler and Lishajko 1961a)

Although the amines in the present experiments were lost from the medullary granules at a slightly higher rate than the ATP, there was no major change in the molar CA/ATP ratio In the nerve granules the ATP loss was very insignificant in comparison with the NA release and thus there was a large exces of adenine nucleotides in these granules towards the end of the incubation

However the lack of parallelism in the NA and ATP loss from the nerve granules may be apparent only since part of the ATP of the nerve granule sediment may well belong to other intraaxonal particles If this is correct, the true ATP level of the specific NA-storing granules would be considerably lower than that found in the whole sediment in which case the NA of the nerve granules under resting conditions would exist in con iderable excess of the total adenine nucleotides

This implies that CA storage by binding to equivalent amounts of adenine nucleotides and protein would apply to only a limited proportion

of the nerve granule amines while the rest would have a different storage mechanism.

Thus the incubation experiments apparently support the concept of non homogeneity of the storage granule amine population.

4 DRUG EFFECTS ON THE SPONTANEOUS RELEASE OF CA AND ATP

Results

In the medullary granules addition of reserpine to the medium at 10^{-7} M—10^{-5} M during incubation at 37°C for 15 min did not cause any deviation from the normal course of release of CA and ATP, as seen in the controls incubated in the absence of the drug (Fig. 4a).

In the nerve granules the same concentrations of reserpine strongly counteracted the fall in NA, thus maintaining a high level of intragranular amines while not altering the spontaneous loss of ATP.

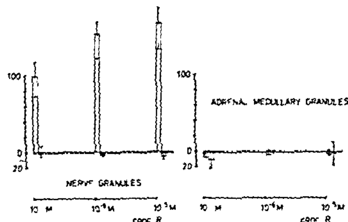
Segontin at least in the two higher concentrations used 3×10^{-5} M and 3×10^{-4} M accelerated the spontaneous fall in both amines and ATP in the medullary granules to about the same extent. At the highest concentration it caused a nearly complete elimination of both (Fig. 4b).

In the nerve granules Segontin in the two lower concentrations 3×10^{-6} M—3×10^{-5} M had a reserpine like effect not changing the spontaneous fall in ATP but strongly counteracting the fall in NA. At the highest concentration 3×10^{-4} M it accelerated the spontaneous fall in both amines and ATP although not quite to the same extent. At this concentration it thus induced changes in the nerve granules similar to those found in the medullary granules.

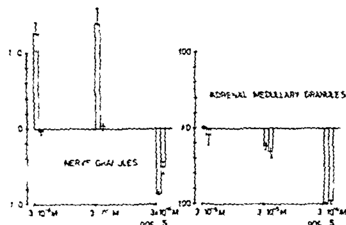
Phenoxybenzamine had opposite effects on the nerve and the medullary granules. At all concentrations tested, from 0.25×10 M to 4×10^{-4} M it accelerated the spontaneous fall in amines and ATP of the medullary granules while at the same concentrations strongly counteracting the fall in NA in the nerve granules and particularly in the highest concentration also that of the nerve granule ATP (Fig. 4c).

Discussion

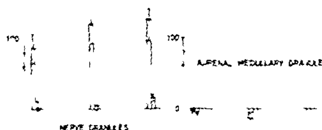
The results obtained with these drugs strongly emphasize the existence of quantitative functional differences between amine storage granules from



a Reserpine (R)
10 ?M 10 M



b Segontin (S)
3 × 10 M 3 × 10 M



c Phenoxybenzamine
PBA 0.25x10 M
4x10 M

Fig. 4 Drug induced deviations from the course of spontaneous decrease in CA and ATP in nerve and adrenal medullary granules on incubation at 37 C for 15 minutes. Ordinates: Per cent deviation from control calculated in the absence of drugs. Abscissae: Drug concentrations. Left column: CA, Right column: ATP. Means and range 3 experiments.

$$\frac{CA_{DRUG} - CA_{CONTROL}}{CA_{CONTROL}} \cdot 100 \text{ and } \frac{ATP_{DRUG} - ATP_{CONTROL}}{ATP_{CONTROL}} \cdot 100$$

different organs They even suggest that the granules may in some respects be qualitatively different, although one hesitates to accept such a conclusion in view of the data indicating the common origin, ontogenetically, of the CA producing and storing systems

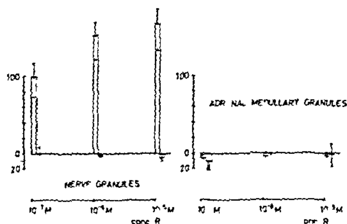
The very existence of drug induced inhibition of the spontaneous loss of CA on incubation *in vitro* indicates that this loss does not represent mere "leakage expressing the degree of damage to the granules but may rather be considered to be a specific process possibly even involving active trans port, as suggested by its temperature dependence

The results from the drug experiments also demonstrate that changes in amines and ATP are much more firmly linked together in the medullary granules than in the nerve granules This again raises the question to what extent the ATP actually measured in the sediment of the nerve preparation represents the ATP of the specific amine storage granules Assuming that the major part of the ATP measured is derived from the storage granules which would be compatible with the concept of an amine ATP complex as essential for amine storage these experiments would imply that the bind ing in such a complex is quite loose and allows the two components to vary independently of each other Alternatively, assuming that only a minor part of the ATP found in the sediment truly belongs to the storage granules, this would mean than only a minor part of the nerve granule amines could be stored by a mechanism requiring equivalence of amines and ATP Thus in either case one must apparently conclude that the role for ATP in the nerve granules is at least quantitatively different from that in the medul lary granules

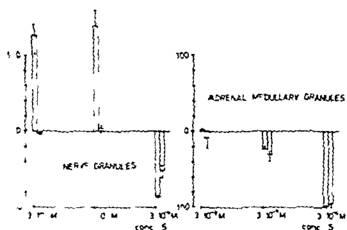
5 AMINE TURNOVER IN THE GRANULES IN VITRO

The rate of uptake of CA from the medium on incubation of nerve (Euler and Lishajko 1963a) and medullary granules (Carlsson et al 1963a) is dependent on the amine concentration in the incubation medium In order to make a fair comparison of the uptake into the two types of granule it was thus necessary to chose appropriate levels of extragranular amines One diffi culty in this connection was the fact that the nerve and medullary granule preparations used in the present series had very different levels of endogenous non granule bound amines of the order of 6 and 150 nanomoles/ml, respec- tively

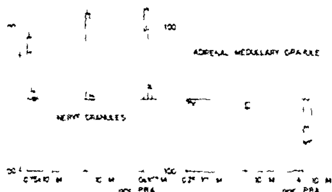
These figures were chosen as reference values Tracer amounts (0 5 μC/ml = 0 14 nanomoles/ml) of H^3 DL-NA and nonlabeled DL- and D NA



a Reserpine (R)
10 M 10 M



b Segontin (S)
3 × 10 ⁶M 3 × 1(



c Phenoxybenzami
PBA 0 25x10
4x10⁻⁴M

Fig 4 Drug ind ed deviations fr m the c urse of pontaneous decrease in CA ATP in nerve and a d enal medullary granules on incubation at 37 C for 1 min Ordinates Per ce t de at on from c ntr ls incubated in th e bsence of dr Abscissae Dr g c n nt at n Left c lumns CA Right c lumns ATP Means and range 3 ex e it ent

$$\frac{CA_{DRUG} - CA_{CONTROL}}{CA_{CONTROL}} \cdot 100 \text{ and } \frac{ATP_{DRUG} - ATP_{CONTROL}}{ATP_{CONTROL}} \cdot 100$$

At 0°C the uptake of amines in the medullary granules was inconspicuous, while it was quite considerable in the nerve granules In both types of granule it was independent of ATP Mg^{++} addition

At 37°C the uptake of amines from the medium was much higher in both the nerve and medullary granules In both types of granule the uptake increased with the amine concentration in the medium Addition of ATP-Mg^{++} caused a marked stimulation of the uptake

However, while the uptake in the nerve granules in the presence of ATP-Mg^{++} resulted in replacement of 25—50 per cent of the endogenous NA with amines derived from the medium the uptake of exogenous amines into the medullary granules under corresponding conditions amounted to only 1.5—4.5 per cent of the amines present in these granules at the beginning of the incubation Thus the turnover rate of the nerve granule amines was, under the present conditions more than ten times higher than that of the medullary granule amines

B UPTAKE AS A FUNCTION OF TIME

Since steady state conditions were approached in the nerve granules at the highest external amine concentration used the intragranular amine level being relatively well maintained throughout the incubation period (Fig 6a) a comparative study of the time course of the uptake in nerve and medullary granules at this concentration level was carried out (Fig 6b) Later experiments established that the initial rate of uptake in the nerve granules was even higher than indicated on the figure After this rapid phase the increase in the degree of "saturation of the nerve granules with exogenous amines as expressed by the specific activity (SA) ratio (SA of the sediment divided by SA of the supernatant), proceeded more slowly During the first half hour the SA ratio failed to reach the value of unity which would indicate that the amines in the extragranular medium were in isotopic equilibrium with the intragranular amines In the medullary granules the rate of uptake was of a different order of magnitude and after a relatively linear initial phase tended to level out at a SA ratio of about 0 13

C UPTAKE IN NERVE GRANULES AS A FUNCTION OF TEMPERATURE

The rapid initial phase of the uptake of amines into nerve granules was studied at 0°, 20° and 37°C in the presence of 96 nanomoles/ml DL-NA and ATP Mg^{++} 3 mM (Fig 7)

The shortest time period studied at 0°C corresponded to the time requi

red for centrifugation 30 min However, there was no difference in the 0°C uptake at 30 and 60 min This uptake may be very rapid possibly almost instantaneous

For the studies at 20° and 37°C the granule preparations were preincubated at the respective temperatures for 4 min in the presence of ATP-Mg^{++} 3 mM to establish temperature equilibrium before the additions of the tracer and non labeled DL-NA were made At both temperatures the rate of uptake leveled off after the initial rapid phase However, there was a large difference in the slope of the uptake curves at the two temperatures a SA ratio of 0.5 being obtained in about 6 min at 37°C while at 20°C about 50 min were required to reach this level

D DRUG INDUCED INHIBITION OF THE UPTAKE

Reserpine is known to inhibit the ATP Mg dependent amine uptake at concentrations from 10 M and upwards *in vitro* both in medullary (Carls

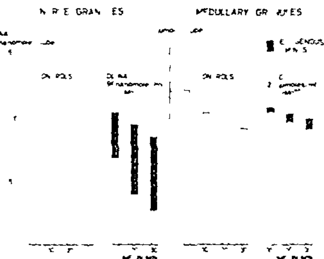


Fig 6a Typical uptake experiment On incubation at 37°C for 30 minutes the CA content of isolated nerve and adrenal medullary granules decreased spontaneously In the presence of H DL-NA 0.5 μC/ml non labeled DL-CA ... nanomoles/ml nerve granules and 2.4 μmoles/ml medullary granules and ATP Mg^{++} 3 mM the spontaneous decrease was ... counteracted This seems to be partly due to inhibition of spontaneous ... but mainly to uptake of amines from the medium ... compensating for the ... loss of endogenous intragranular CA The data from this experiment are included in Fig 6

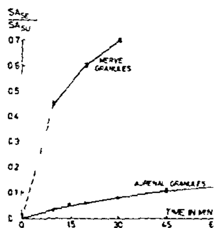


Fig 6b Time course of uptake of DL-CA from the medium into isolated nerve and medullary granules on incubation at 37°C in the presence of H DL-NA 0.5 μC/ml non labeled DL-CA 96 nanomoles/ml nerve granules 2.4 μmoles/ml medullary granules and ATP Mg^{++} 3 mM Ordinate Specific activity (SA) of the sediment (SED) expressed as CPM/nanomole divided by SA of the supernatant (SU)

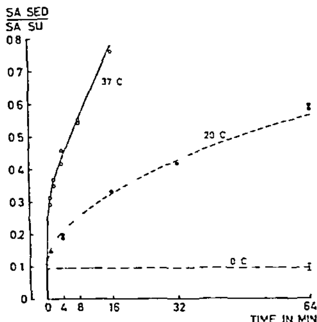


Fig 7 Time course of uptake in nerve granules of exogenous DL-NA on incubation at 0 C, 20 C and 37 C in the presence of H^3DL-NA 0 5 µC/ml non labeled DL-NA 96 nanomoles/ml and ATP Mg^{++} 3 mM The 20 C and 37 C tubes were preincubated with ATP Mg^{++} for 4 minutes at the respective temperatures before the CA additions were made

son et al 1962 1963a Kirshner 1962) and in nerve granules (Euler and Lishajko 1963b) while a 10^{-3}M concentration of the drug was required to produce inhibition of uptake in the amine containing particulate fraction isolated from the rat heart (Potter and Axelrod 1963b)

In nerve granules previously partially depleted by preincubation the amine uptake occurring at A or NA concentrations in the medium of 60—120 nanomoles/ml was not blocked by reserpine addition (Euler and Lishajko 1963b) This was interpreted as indicating that the primary action of reserpine is not to inhibit amine uptake but rather to block the spontaneous release thus leaving no binding sites available for exogenous amines However preincubation with reserpine at 20°C for 1 hour or at 0 C for 24 hours led to an essentially complete inhibition of the uptake of amines into the nerve granules even after previous partial depletion Thus reserpine when acting over longer periods of time was assumed to "interfere with some factor necessary for the fixation of the CA to the granules (Euler and Lishajko 1963b)

In the present experiments the object was to establish the dose dependence of the reserpine induced inhibition of amine uptake and to find out whether it was competitive or non-competitive Thus reserpine was

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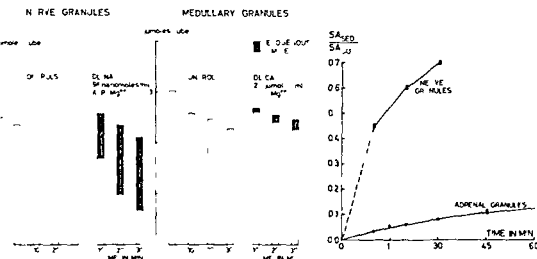


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Fig 6b Time course of uptake of DL CA from the medium into isolated nerve and medullary granules on incubation at 37 C in the presence of H DL NA 0 5 μC/ml non labeled DL CA 96 nanomoles ml nerve granules) 2 4 μmoles/ml (medullary granules) and ATP Mg 3 mM Ordinate Specific activity (SA) of the sediment (SED) expressed as CPM/nanomole divided by SA of the supernatant (SU)

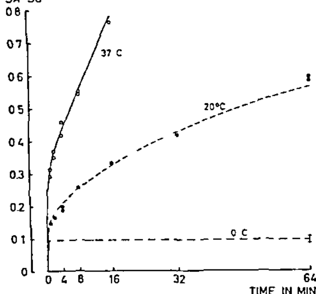


Fig. 7 Time course of uptake in nerve granules of exogenous DL-NA on incubation at 0° C, 20° C and 37° C in the presence of H^3DL-NA 0.5 μC/ml, non labeled DL-NA 0.6 nanomoles/ml and ATP-Mg^{++} 3 mM. The 20° C and 37° C tubes were preincubated with ATP-Mg^{++} for 4 minutes at the respective temperatures before the CA additions were made.

son et al. 1962, 1963a, Kirshner 1962) and in nerve granules (Euler and Lishajko 1963b), while a 10^{-3} M concentration of the drug was required to produce inhibition of uptake in the amine containing particulate fraction isolated from the rat heart (Potter and Axelrod 1963b).

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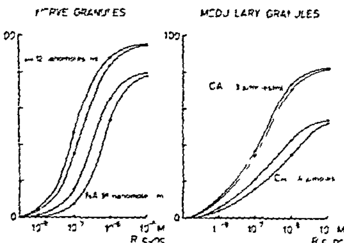


Fig 8 Inhibitory effect of reserpine (R) 10^{-9}M–10^{-5}M on uptake of exogenous CA in isolated nerve and medullary granules on incubation at 37 C for 15 minutes in the presence of ^{3}H DL-NA 0.5 μC/ml two different concentrations of non labeled DL-CA 12 or 96 nanomoles/ml (nerve granules) and 0.3 or 2.4 μmoles/ml (medullary granules) and ATP Mg + mM

Ordinates Per cent inhibution of uptake ^{3}H DL-NA in sediment caused by reserpine
Abscissae Reserpine concentrations
Data from 2 experiments

$$100 - \frac{CIM_{RESERPINE}}{CIM_{CONTROL}} \times 100$$

added to the medium at the start of the incubation period in concentrations from 10^{-9}M to 10^{-5} M The medium further contained 0.5 μC/ml of H^{3} DL-NA ATP Mg 3 mM and a total of non labeled DL-CA of 12 or 96 nanomoles ml in the nerve granule suspension and 0.3 or 2.4 μmoles ml in the medullary granule suspension

The results are presented in Fig 8

Both in the nerve and medullary granules incubated at 37 C for 15 minutes the amine uptake was progressively inhibited with increasing reserpine concentrations in the medium The inhibition was barely visible at reserpine 10^{-9}M but at 10^{-5} M and in the lower amine concentration it was nearly complete both in the nerve (93 per cent inhibition) and in the medullary granules (83 per cent) At the higher amine concentrations the inhibition was less complete even at the highest reserpine concentration both in the nerve (76 per cent) and medullary (54 per cent) granules

In the nerve granules 50 per cent inhibition of the uptake was observed at reserpine concentrations of 2×10^{-7} M and 7×10^{-7} M for the lower and higher amine concentrations respectively This corresponds to a molar ratio CA reserpine of 60 1 and 140 1 which implies that one reserpine molecule was able to successfully cope with from 60 to 140 CA molecules trying to enter the nerve granules

In the medullary granules 50 per cent inhibition was obtained at reserpine 3×10^{-7} M and 3×10^{-6} M for the two amine concentrations used In

this case the molar CA/reserpine ratio at 50 per cent inhibition was even larger, 1000 1 and 400 1 indicating that the relative inhibitory efficiency of reserpine may be even higher in this type of granule

Discussion

Since the tracer used in the present series was racemic, non labeled DL-NA rather than the physiologically occurring L-form was added to the medium, when higher concentrations of amines were needed in this Thus the distribution of the tracer should distinctly mirror that of the larger population of non labeled amines added to the medium

Recent observations both with isolated nerve (Euler et al 1963b, Euler and Lishajko 1964) and medullary granules (Carlsson et al 1963a) and in the amine containing particles from the rat heart (Potter and Axelrod 1963b), as well as *in vivo* (Kopin and Bridgers 1963, Maickel et al 1963 Iversen 1963) indicate the existence of a certain degree of stereospecificity in CA uptake and storage mechanisms Thus conclusions based on the absolute values obtained in the present series with the use of DL-amines should be used with caution in application to *in vivo* physiology However experiments with C^{14} L-NA[1] diluted with non labeled L-NA to the same total amine concentrations as those used in the present series, showed the same general trend as that found with the racemic mixture although the absolute values were higher when the L-isomer was used alone (Euler, Lishajko and Stjarne to be published)

In the present experiments only NA uptake was studied on the premise that the uptake mechanisms in nerve (Euler and Lishajko 1963a) and medullary granules (Carlsson et al 1963a) do not show distinct preference to either A or NA

With the aid of labeled DL-NA it was possible to demonstrate striking differences in the relative uptake of exogenous amines into nerve and medullary granules under the experimental conditions used

In the presence of the highest amine concentrations in the medium "steady state conditions were approached the total amount of amines in the granules being largely unchanged or only slightly decreased during the periods observed The amount of exogenous amines taken up under these conditions does not express net uptake, but rather exchange of endogenous amines with amines from the medium and may thus be regarded as a

[1] Kindly put at our disposal by Dr I J Kopin National Institutes of Health Bethesda Maryland

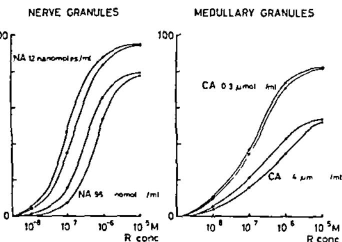


Fig 8 Inhibitory effect of reserpine (R), 10 ⁸M 10 ⁵M on uptake of exogenous CA into isolated nerve and medullary granules on incubation at 37 C for 15 minutes in the presence of H³DL NA 0 5 µC/ml two different concentrations of non labeled DL-CA 12 or 96 nanomoles/ml (nerve granules) and 0 3 or 2 4 µmoles/ml (medullary granules) and ATP Mg⁺⁺ mM

Ordinates Per cent inhibition of uptake of H³DL NA in sediment caused by reserpine ¹
Abscissae Reserpine concentrations
(Data from 2 experiments)

$$^{1}100 \quad \frac{CPM_{RESERPINE}}{CPM_{CONTROL}} \times 100$$

added to the medium at the start of the incubation period in concentrations from 10 ⁸M to 10 M The medium further contained 0 5 μC/ml of H³ DL-NA ATP-Mg , 3 mM, and a total of non labeled DL-CA of 12 or 96 nanomoles/ml in the nerve granule suspension and 0 3 or 2 4 μmoles/ml in the medullary granule suspension

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In the nerve granules 50 per cent inhibition of the uptake was observed at reserpine concentrations of 2 × 10 M and 7 × 10 M for the lower and higher amine concentrations, respectively This corresponds to a molar ratio CA/reserpine of 60 1 and 140 1 which implies that one reserpine molecule was able to successfully cope with from 60 to 140 CA molecules trying to enter the nerve granules

In the medullary granules 50 per cent inhibition was obtained at reserpine 3 × 10 ⁷M and 5 × 10 M for the two amine concentrations used In

appear necessary to postulate a direct "releasing" action on the granules for agents mobilizing the neurotransmitter from the noradrenergic nerve terminals (Euler 1962 Stjarne 1964)

From the pharmacological point of view, one may conclude that any agent which is able to cause complete selective inhibition of the uptake of amines into the granules without affecting spontaneous outflux, would cause rapid depletion of the granules according to the present data a 50 per cent depletion in less than 10 minutes One may also conclude that a drug with the abovementioned effect ought to cause CA depletion in nerve tissue more than ten times as rapidly as in the adrenal medulla, a time relation which applies to drugs like reserpine (Carlsson et al 1957a)

6 PERMEABILITY OF THE NERVE GRANULE MEMBRANE TO CA

These experiments which were restricted to nerve granules were carried out at 0°C The total water content of the nerve granule sediment was determined by measuring the tritiated water space The uptake of H^3 DL-NA in the presence of low (12 nanomoles/ml) and high (96 nanomoles ml) concentrations of non labeled DL-NA, with and without addition of reserpine 10 M was also studied in other aliquots of the same granule preparation

The results of the experiments are summarized in Table III and Fig 9 Even at 0°C a considerable amount of exogenous amines was always found in the washed and resuspended sediment Since this occurred in the absence of net increase in the total NA fluorimetrically determined the presence of exogenous NA in the granules must be due to exchange with endogenous amines When constant amounts of labeled NA were added to aliquots of nerve granules in the presence of increasing amounts of non labeled NA the uptake of the radioactive amine in the granules was competitively inhibited In the presence of reserpine a dose dependent decrease in the amount of exogenous amines in the granules was seen, reaching a maximum inhibition of about 50 per cent at reserpine 10^{-5} M Addition of ATP or EDTA either alone or in combination was not inhibitory, but possibly even slightly stimulated the 0°C uptake

The total H^3 DL-NA radioactivity recovered in the wash after resuspension of the granule sediment was too large to be accounted for by amines dissolved in trapped intergranular water but closely agreed with the amount calculated to be present in the total tritiated water space as

Table III *Study of the permeability of the nerve granule membrane* Cf Fig 9

A The total water in the nerve granule sediment pellet was determined as the tritiated water (T_2O) space

An aliquot of the nerve granule suspension was incubated with 0 8 ml of T_2O (Packard 2 51 dpm/ml) for 30 minutes at 0 C The distribution of the T_2O radioactivity between the washed non resuspended sediment (SED) supernatant (SU) and wash fluid (WASH) was determined

The tritiated water space of the sediment pellet was calculated as follows

$$T_2O \text{ space} = \frac{CPM^T_{SED} + CPM^T_{WASH}}{CPM^T_{TOT}} \times \text{volume}_{tube}$$

T_2O radioactivity in SED WASH and SED+SU+WASH is expressed as CPM^T_{SED} CPM^T_{WASH} and CPM^T_{TOT} respectively

B The distribution of tritiated DL-NA between the washed and resuspended sediment the supernatant and the wash fluid on incubation of aliquots of the nerve granule suspension at 0 C for 30 minutes was studied

All tubes contained a constant amount of H^3DL-NA while nonlabeled DL-NA was added at two different concentrations 12 and 96 nanomoles/ml respectively The incubations were carried out in the absence (C) and in the presence (R) of reserpine 10⁻ M

The tritiated NA radioactivity in the total pellet water was calculated as follows

$$CPM^{NA} \text{ in } T_2O \text{ space} = \frac{CPM^{NA}_{SU}}{\text{volume}_{tube}} \times T_2O \text{ space}$$

	Tube no	ADDITIONS H^3DL-NA ml	Non-labeled DL-NA nanomoles ml	Reserpine	T_2O ml	Vol in tube ml	RADIOACTIVITY IN CPM SED	SU	WASH	EXOG NA IN SED nanomoles
A	1	—	—	—	0 8	7 60	474	291000	247	—
B	2	0 1	12	—	—	7 15	4300	1121000	3300	0 327
	3	0 1	12	10⁻ M	—	7 20	1810	1150000	3275	0 136
	4	0 1	96	—	—	7 25	2910	1180000	3650	1 710
	5	0 1	[illegible]	10⁻ M	—	7 30	1590	1190000	3160	0 910

$$T_2O \text{ space} = \frac{474 + 247}{474 + 247 + 291000} \times 7\ 8 = 0\ 0193 \text{ ml}$$

$$CPM^{NA} \text{ in } T_2O \text{ space}^1 = \frac{1160250}{7\ 225} \times 0\ 0193 = 3100$$

Mean values for CPM^{NA}_{SU} and for volumes were used

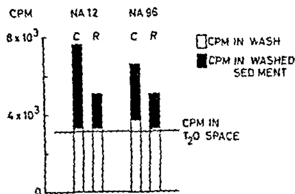


Fig 9 Comparison of radioactivity due to H^3DL-NA found in washed sediment and in wash with radioactivity calculated to be present in the total water space of the nerve granule sediment pellet

One aliquot of a nerve granule suspension was used for determination of the tritiated water (T O) space (for details see Table III)

Other aliquots were incubated at 0 C for 30 minutes with constant amounts of H^3DL-NA in the presence of non labeled DL NA 12 or 96 nanomoles/ml and in the absence (C) and presence (R) of reserpine 10- M For details concerning the calculations see Table III

suming equal amine distribution on both sides of the granule membrane The radioactivity found in the wash was independent of the amount of non labeled amines present, or by the addition of reserpine

Discussion

The membrane of the adrenal medullary granules has been reported to be permeable to CA (Carlsson and Hillarp 1958, Hillarp 1959 Kirshner 1964)

The obvious conclusion from the present experiments is that the nerve granule membrane too is permeable to NA at 0°C, under the experimental conditions used The major part of the exogenous amines entering the nerve granules, probably by passive diffusion could be removed by washing and resuspension However amines were to some extent bound in the nerve granules even at 0°C by a mechanism which protected them against removal by washing

The 0°C amine uptake occurring in bovine medullary granules (Carlsson et al 1963a) and in isolated rat heart granules (Potter and Axelrod 1963b) was regarded as an unspecific adsorption However the uptake at this temperature observed in the present experiments showed several features, which are difficult to explain using the adsorption hypothesis

Thus the uptake did not lead to a net increase in the total nerve granule

amin s determined fluorimetrically, implying that it was due to exchange with endogenous intragranular NA. Moreover, the accumulation of amines from the medium in the nerve granules at 0 C was markedly inhibited by low concentrations of reserpine.

The possibility that at least part of this uptake may be specific and physiologically significant must be kept in mind. It may represent binding of the amines to the transport system which in the presence of active metabolism and at higher temperature transfers the amines to the interior of some storage structure in the granules. From that point of view the observation that reserpine competitively inhibited this step may provide vital information concerning the mechanism whereby this drug blocks amine transport.

The finding that the nerve granule membrane is permeable to CA does not invalidate the observations indicating the existence of specific amine uptake and release mechanisms in these granules. The facts that exogenous amine uptake was clearly stimulated by ATP-Mg, that this uptake was temperature dependent and definitely inhibited by a number of drugs including reserpine at very low concentrations, and that spontaneous loss of endogenous nerve granule amines was inhibited by the same drugs at the same low concentrations strongly support the concept of specificity for the processes of nerve granule amine uptake, storage and release.

However, the permeability of the nerve granule membrane to the very material the granules specifically store indicates that passage at this level is governed by physicochemical forces of low specificity. Thus attention has to be directed to other structures in the granules which must be responsible as anatomical substrate for specific uptake and release of the amines. It must by elimination be quite safe to conclude that the processes [illegible] strongly and characteristically affected by addition of various potent drugs as demonstrated in the present investigation take place in the [illegible] of the nerve granules.

General Discussion

The [illegible] experiments have demonstrated several quantitative and possibly qualitative differences between bovine noradrenergic nerve and adrenal [illegible] granules.

In [illegible] with previous reports (Falck et al 1956, Hasselblo et al 1956, [illegible], Euler et al 1962a) the relative proportions of CA

and ATP were found to be of the same order of magnitude in the two types of granule This finding is compatible with the hypothesis that the amines are bound in the storage granules by a mechanism requiring molar equivalence amines to adenine nucleotides (Hillarp 1958b)

However, in the medullary granules the total amount of adenine nucleotides was insufficient to allow this type of binding of the entire amine population Thus at least a minor fraction of the amines obviously must be stored by some different mechanism independent of adenine nucleotides (cf Hillarp 1960a)

In the medullary granules the relative changes in the amines and ATP were almost parallel during spontaneous release on incubation at 37°C or as the result of drug addition However lowering of the osmotic pressure caused a rapid and complete depletion of the ATP while part of the amines remained in the granules Thus the lysis experiments strongly suggest that a considerable proportion of the medullary granule amines is stored independently of ATP Osmotically induced damage to the structure of the medullary granules apparently allowed the amines which were bound in a water soluble form possibly as a complex CA ATP water soluble protein to diffuse out of the granules, while the amines bound to the stroma without equivalent amounts of adenine nucleotides were largely retained

The study of the fate of the medullary granule ATP on graded lysis was complicated by the fact that incubation at 0°C with the technique described in the presence of a strong anion exchange resin resulted in a considerable reduction of the ATP and CA content of the granules even in isotonic media The loss of ATP in the granules on addition of ion exchange resin may be non specific, due to mechanical damage or to removal of essential anions However the ATP loss may also imply that this compound is located superficially enough in the granules to be accessible to the resin acting on the granule surface From that point of view, the lack of similar effects on the CA on addition of aluminium oxide indicates that the amines may be stored in a different way inaccessible to adsorbing forces acting on the surface of the granules

In the nerve granules the much stronger release of NA than of ATP on incubation at 37 C clearly shows that independent amine release with retention of the ATP may occur in these granules

This is of interest in view of the recent report (Potter and Axelrod 1963b) that reserpine treatment of rats *in vivo* did not significantly reduce the ATP of the isolated amine storage granules from the heart while strongly reducing their amine content

Similar independent variations of CA and ATP were observed in the

nerve granules in the present investigation on addition of drugs to the incubation medium Thus inhibition of spontaneous amine release was observed both in the absence of effect on ATP release and in the presence of partial inhibition of ATP release Acceleration of amine release was accompanied by increased ATP loss, although not quite to the same extent

There is considerable evidence that ATP is concerned with CA storage both in the nerve and medullary granules The present results do not exclude the possibility that a large proportion of the amines in both types of granule may be stored in a water-soluble complex with protein and equivalent amounts of adenine nucleotides This postulated storage structure has never been directly demonstrated and is thus still hypothetical Recently some indirect evidence supporting the feasibility of such a complex has appeared Thus it was claimed that addition of ATP to an A solution in heavy water resulted in a change of the nuclear magnetic resonance spectrum of A indicating a two-point stabilization of the A molecule, across the hydroxyl and amino groups of the side chain (Weiner, Pappas and Jardetzky 1961) Moreover, studies of the water soluble protein from bovine adrenal medullary granules isolated by density gradient centrifugation, indicated that addition of ATP Mg^{++} alone or in combination with A resulted in a change in the physical properties of the protein i e in its sedimentation properties In starch gel electrophoresis of a mixture of protein and A, part of the A behaved as a cation and moved to the cathode while some moved together with the protein to the anode (Blaschko and Helle 1963)

However this cannot be the exclusive amine storage mechanism A considerable proportion of the amines in both types of granule appear to be stored in a water insoluble state, probably bound to lipid or lipoprotein material in the stroma

Different distribution of the intragranular amines over two or more types of storage mechanisms may possibly be the basis of the striking differences in the effects of the drugs tested in the present experiments on nerve and medullary granules This view would be compatible with the concept of common ontogenetical origin of the cells containing the granules the noradrenergic axones and adrenal medullary cells

The locus of action of the drugs which at low concentrations inhibit the spontaneous amine release and uptake mechanisms in the granules is probably separate from the granule membrane The fact that a drug like reserpine is able to succesfully block the uptake of amines occurring the medium at from 60 140 nerve granules) and 400—1000 medullary granules) times higher molar concentration definitely suggests that it acts at some "bottle neck in the specific transport systems of the granules As previously

mentioned this strategic site is probably located in the stroma of the interior of the granules.

The experiments with labeled NA clearly demonstrate the large difference in amine turnover rate in nerve and medullary granules. This may well be the basis of the differences in the time course and completeness of the CA depletion induced in sympathetically innervated tissues and adrenal medulla by various drugs. Furthermore, in view of the rapid spontaneous turnover rate in the nerve granules it does not appear necessary to postulate any direct "releasing" action on these granules to explain the mobilization of the sympathetic neurotransmitter from the axone, resulting from the depolarization of the axonal membrane or from the administration of drugs like reserpine or TA.

The results of the study of the membrane permeability in the nerve granules with tritiated water and labeled NA imply that amines present in the extragranular medium will rapidly equilibrate by diffusion throughout the total granule water space. The next step apparently is non-energy consuming and probably consists in binding to some site on the surface of the intragranular structures by exchange with endogenous amines. This binding appears to be firm enough to make the amines resistant to washing and thus turns them functionally water insoluble. The binding site may well be the mechanism whereby the specific transport system of the granules capture amines, which will then be transported to the interior of the granular "substructure" by an energy requiring process. It is conceivable that the interior of this "substructure", which presumable has a low water content, may represent the part of the granules where amines occur bound in a complex with ATP and protein. The boundaries of the "subgranules" appear to be poorly permeable to the amines, and in- and outward transport is probably specific and possibly coupled. Outward transport thus also requires an initial step consisting in binding to the transport system.

Using this hypothetical model (Fig. 10) of the nerve storage granule it is possible to provide rational explanations of several of the experimental data. Thus it accounts for the 0°C appearance of exogenous amines in the granule water in equilibrium with the amines in the external medium. It also explains the rapidly saturated and amine concentration dependent binding by exchange at 0°C. It interpretes the temperature dependance of the penetration of exogenous amines into the interior of the granules in terms of metabolic activation of an energy requiring active transport mechanism. The acceleration of the uptake in the presence of ATP-Mg^{++} would be due to increased energy supply to this transport system. It explains the inhibition of amine uptake induced by reserpine both at 0°C and 20°C or

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However, this cannot be the exclusive amine storage mechanism. A considerable proportion of the amines in both types of granule appear to be stored in a water insoluble state, probably bound to lipid or lipoprotein material in the stroma.

Different distribution of the intragranular amines over two or more types of storage mechanisms may possibly be the basis of the striking differences in the effects of the drugs tested in the present experiments on nerve and medullary granules. This view would be compatible with the concept of common ontogenetical origin of the cells containing the granules, the noradrenergic axones and adrenal medullary cells.

The locus of action of the drugs which at low concentrations inhibit the spontaneous amine release and uptake mechanisms in the granules is probably separate from the granule membrane. The fact that a drug like reserpine is able to successfully block the uptake of amines occurring, the medium at from 60–140 (nerve granules, and 400–1000 medullary granules) times higher molar concentration definitely suggests that it acts at some "bottle neck" in the specific transport systems of the granules. As previously

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stored in two or more different ways. It is conceivable that the major part of the medullary granule CA is stored in a complex with adenine nucleotides and water soluble protein. This type of storage complex may exist to some extent in the nerve granules as well. However, in these granules the majority of the CA appear to be stored by a mechanism which renders them functionally water insoluble, possibly by some kind of binding to lipid or lipoprotein material in the stroma. This may also apply to the smaller CA pool in the medullary granules, which seems to be stored independently of adenine nucleotides.

One of the more striking quantitative differences between nerve and medullary granules was the more than ten fold difference in CA turnover. In the nerve granules the exchange of endogenous for exogenous CA proceeded so fast that one half of the amine molecules were renewed in less than 10 minutes, while in the medullary granules at most 10—15 per cent of the endogenous CA were exchanged for amines from the medium during the first hour. In both types of granule a certain uptake of exogenous amines occurred at 0 C. This was more closely studied in the nerve granules, where it proved to be quite considerable.

This low temperature uptake does not represent net uptake but exchange. It was markedly inhibited by low concentrations of reserpine. Thus it is possible that it may be to some extent specific and represent binding to the transport system of the granules.

The conclusions concerning the dynamics of uptake, storage and release of CA in the granules and the mechanism of the action of reserpine and ATP-Mg are summarized in a diagram.

CHAPTER III

TIME COURSE OF THE UPTAKE AND ELIMINATION OF H^3DL-NA IN THE HEARTS OF NORMAL AND RESERPINE-TREATED RABBITS

Introduction

In the preceding section results obtained in experiments with isolated granules from bovine splenic nerves and adrenal medulla were presented In this section two issues emerging from the *in vitro* study will be dealt with in experiments *in vivo*

Turnover of amines

In isolated nerve granules the amine turnover rate was found to be so high that one half of the endogenous NA was replaced by exogenous NA from the incubation medium in less than 10 minutes The value for the *in vitro* turnover rate of nerve granule amines thus obtained was of a different order of magnitude from those calculated for amines in different sympathetically innervated organs on the basis of *in vivo* studies of the rate of disappearance of labeled amines from the tissue in question (Montanari et al 1963 Spector et al 1963) However the discrepancy was assumed to be apparent only and due to differences in the parameters actually measured

The in vitro results in this investigation and evidence from the literature, suggested that the highly efficient uptake mechanisms both in the specific granules inside the axone and in the axonal membrane normally recapture the major part of the neurotransmitter continuously or intermittently released into the surrounding medium This was assumed to be the explanation of the much higher rate of *appearance* of tracer amounts of labeled amines in the tissues than of their subsequent *disappearance* (Axelrod et al 1959 Whitby et al 1961 Montanari et al 1963)

In order to compare the amine turnover in the whole organ with that of the specific granules *in situ* the time course of the appearance and subsequent disappearance of H^3DL-NA was studied both in the rabbit heart as a whole and in the subcellular fraction containing the major part of the specific amine granules i e the high speed sediment obtained by differential centrifugation

The mechanism of action of reserpine

The exact mechanism of the local effect of reserpine leading to CA depletion is as yet poorly understood. *In vitro* results clearly show that reserpine concentrations, which from the *in vivo* pharmacological point of view must be regarded as excessively high, are required to produce release of amines by a direct action on the isolated granules from bovine noradrenergic nerves (Euler and Lishajko 1961c), from the rat heart (Potter and Axelrod 1963b) or from bovine or rabbit adrenal medulla (Stjärne and Schapiro 1959, Euler and Lishajko 1961c, Weil-Malherbe and Posner 1963). The low concentrations of reserpine, which may be expected to occur *in vivo* on administration of a moderate dose of the drug, strongly counteract the spontaneous release of amines from the isolated nerve granules (Euler and Lishajko 1961c). Thus the *in vitro* evidence indicates that reserpine not only is devoid of power to deplete the granules, but actually exerts the opposite effect, delaying the spontaneous decrease of the intragranular amines.

However reserpine has been found to have other *in vitro* effects on the isolated granules, which may possibly explain the CA depleting effect of this drug *in vivo*. Thus concomitantly with the "protective" effect on the nerve granule amines, reserpine *in vitro* also strongly inhibited CA uptake into these granules (Euler and Lishajko 1963b) as well as into the granules from the adrenal medulla (Carlsson et al. 1962, 1963a, Kirshner 1962). If this applies *in vivo* the result would be increased exposure of the amines to intracellular enzymatic destruction, and also decreased rate of synthesis of NA, since the β-hydroxylation of DA takes place in the granule fraction (Kirshner 1957, Potter and Axelrod 1963b). Both mechanisms obviously would lead to CA depletion of the tissues.

Considerable experimental evidence supports the concept that increased enzymatic destruction of the NA in the axones is a major factor in the mechanism of action of reserpine on the NA stores in the tissues. Thus the reserpine induced depletion of the neurotransmitter in the noradrenergic axones is normally accompanied by few signs of increased discharge of intact NA from the axones. Only after inducing supersensitivity to NA by using spinal animals (Schneider and Rinehart 1956, Maxwell et al. 1957), ganglionic blockade (Maxwell et al. 1957, Beck, Hilton and Domino 1957, Domino and Rech 1957) or cocaine treatment (Horita 1958) did the administration of reserpine result in clear cut signs of increased peripheral sympathetic "tone".

While reserpine did induce increased secretion of CA from the adrenal

medulla of the rabbit (Kroneberg and Schumann 1958) and the cat (Stjarne and Schapiro 1958), it did not cause any considerable increase in the output of intact NA from noradrenergically innervated tissues as the dog heart (Harrison et al 1963) Nor did it cause any consistent increase in the urinary excretion of intact NA (Carlsson et al 1957a)

On the other hand reserpine treatment resulted in increased output of deaminated metabolites from the isolated rat heart (Kopin et al 1962 Nash et al 1963) and in the appearance of increased amounts of such metabolites in the rat urine (Kopin and Gordon 1962, 1963a)

These data strongly suggest that the neurotransmitter disappearing from the noradrenergic axones as a result of reserpine treatment, is largely deaminated intraaxonally prior to departure from the axone (Carlsson et al 1957a Kopin and Gordon 1962) This view is further supported by the observation that pretreatment with mono-amine oxidase inhibitors partly prevents the depletion of the NA stores normally induced by reserpine (Carlsson et al 1957a, Pletscher et al 1959)

There is also considerable evidence that inhibition of the uptake of amines into the granule fraction of the adrenal medulla is a characteristic feature accompanying the fully developed functional paralysis of the peripheral noradrergic system resulting from reserpine administration Thus reserpine pretreatment was shown to inhibit the *in vivo* incorporation in the granule fraction of rabbit adrenal medulla of DA or NA formed on injection of large amounts of L-DOPA (Bertler Hillarp and Rosengren 1961) or of 5 hydroxytryptamine formed on administration of large amounts of 5 hydroxytryptophane (Carlsson, Jonasson and Rosengren 1963) Similarly reserpine pretreatment resulted in strong inhibition of the *in vitro* uptake of C^{14}DA or C^{14} A in isolated granules from the cat (Kirshner et al 1963) and the rabbit adrenal medulla (Lundborg 1963)

However according to the data presented by the investigators mentioned above the development of clear-cut inhibition of the uptake into the granule fraction from the adrenal medulla requires a considerable time Thus the latency after the administration of reserpine until a 50 per cent inhibition of the uptake is established appears to be of the order of 6 hours But the time required to obtain a 50 per cent depletion of the rabbit heart or brain is of the order 20 minutes (Carlsson et al 1957a) This may possibly be explained by differences in the permeability to reserpine of the membranes of the adrenal medullary cells and the noradrenergic axones Alternatively it may indicate that the action of reserpine is not directed exclusively to the intracellular granules

The possibility that the primary action of reserpine leading to the rapid

depletion of sympathetically innervated tissues as the brain or heart, may be exerted at the level of the axonal membrane has in fact been proposed by several authors (Weil Malherbe, Posner and Bowles 1961, Dengler et al 1962 Campos and Shideman 1962)

Regardless of whether the effect is exerted at the level of the intracellular granules or at the cell membrane or at both levels the uptake of circulating amines in the tissues was with few exceptions (see below), found to be strongly reduced after reserpine treatment (Muscholl 1960, Hertting et al 1961, Crout et al 1962, Andén et al 1963, 1964)

Recently the importance of the time factor in studies of the reserpine-induced inhibition of amine uptake has been emphasized Thus on the basis of analysis of the time course of the inhibition of amine uptake *in vitro* and *in vivo* in the granule fraction of the rabbit adrenal medulla after reserpine treatment, it was pointed out that restoration of the amine uptake at this level showed a much closer correlation to clinical recovery from the signs of paralysis of the peripheral noradrenergic system, than did the restoration of a normal content of NA in the tissues (Lundborg 1963, Carlsson et al 1963b) Similar results were obtained in a study where the time course of the inhibition of uptake of H^3DL NA in the heart and in skeletal muscle of the rat, treated with reserpine was directly compared with the time course of depletion and repletion of the NA content in these organs and with the appearance and subsequent disappearance of signs of functional paralysis of the noradrenergic innervation of the eye (Andén et al 1964) On the basis of these studies it was proposed that only a minor fraction of the NA normally contained in the stores of the axones is in any given moment essential for impulse transmission at the noradrenergic neuroeffector junction and that the neurotransmitter release triggered by the depolarization of the axonal membrane occurs from a labile fraction in the specific intraaxonal granules (Carlsson et al 1963b Lundborg 1963 Andén et al 1964 Carlsson 1964)

In the present experiments two aspects of the problem of the mechanism of the action of reserpine were investigated In the first series the time course of the depletion and the initial phase of the repletion of the NA in the rabbit heart after a single intravenous dose of reserpine was studied in parallel with the time course of the inhibition of uptake of circulating H^3DL-NA in the heart In the second series the mechanism of the early inhibitory effects of reserpine on NA uptake in the rabbit heart was studied by chromatographic analysis of the changes in total radioactivity and in the pattern of tritiated NA metabolites induced by reserpine pretreatment

Methods

55 rabbits of mixed strains and both sexes, weighing 2 5—3 5 kg were used for the investigation

In the first series untreated rabbits received an intravenous injection in the marginal vein of the ear of 82 μC/kg (11 6 nanomoles/kg) of H^3DL NA and were killed by air embolism after intervals varying from 1 to 1024 minutes

In the second series the rabbits were given an intravenous injection of reserpine (0 25 mg/kg) At time intervals varying from 1 to 4096 minutes after this, they received the standard H^3DL-NA dose and were killed 16 minutes later

In the third series 34 or 82 μC/kg of tritiated NA was injected 64 minutes after reserpine These rabbits and non reserpine treated controls were killed 1 or 4 minutes later

All steps from this point were carried out at ice water temperature

The heart was rapidly dissected out In the first two series the coronary tree was flushed with approximately 40 ml of Tyrode s solution containing reserpine 5×10^{-6} M using a syringe and adapter loosely fitting into the cut aorta The reserpine was added to inhibit uptake of tritiated NA in the particulate fractions of the heart during the homogenization procedure The heart cavities were opened and carefully rinsed from blood The heart was then in the first two series transferred to 30 ml of isotonic potassium phosphate buffer pH 7 5 containing EDTA 2 mg/ml weighed minced with scissors and homogenized using an Ultra Turrax apparatus operated for 15 seconds at slow speed and for 15 seconds at top speed This homogenate was then centrifuged as described below

In the third series, which was specifically intended for chromatographical analysis of the metabolite pattern the flushing of the coronary tree was omitted and no fractionation was carried out After opening the cavities and carefully rinsing the blood from them the heart was weighed minced and homogenized in 25 ml of 5 per cent trichloroacetic acid

The homogenates from the first two series were centrifuged for 10 minutes at 5000 x *g* The supernatant was decanted and the sediment resuspended in 20 ml of buffer and recentrifuged at the same speed This led to the formation of the final slow speed sediment The pooled supernatants were recentrifuged at 100 000 x *g* for 30 minutes This supernatant was decanted and the high speed sediment was washed by resuspension in 32 ml of buffer and recentrifuged at 100 000 x *g*

The three fractions, the washed low and high speed sediments and the high speed supernatant, were extracted with 5 per cent and 10 per cent trichloroacetic acid, respectively.

In the trichloroacetic acid extracts the precipitate was removed by filter suctioning or by centrifugation. The precipitate was washed and the extract was then in the first two series purified by adsorption on aluminium oxide at pH 8.3 and eluted with acetic acid according to Euler and Lishajko (1961b).

In the third series, the crude trichloroacetic acid extract was used for chromatography. Non-labeled NA and normetanephrine (NM), 50 μg of each, were added to the extract which was adjusted to pH 5 with potassium hydroxide. An aliquot of this solution was chromatographed as described below.

CA assay. The CA were determined fluorimetrically as previously described. The recovery of labeled and unlabeled NA added to the trichloroacetic acid extract and then carried through the purification procedure was 79.2±1.7 per cent.

Radioactivity determination. Radioactivity was determined using a Tri-Carb Liquid Scintillation Spectrometer as previously described.

Ion exchange chromatography

The chromatographic analysis was carried out using Amberlite CG 120 Type 2 (200—400 mesh) according to Häggendal (1963). The column size was 0.4×150 mm. Gravity flow at a rate of about 4 ml/hour was used. 0.5 M HCl was used as eluant for the first 30—40 ml, then the eluant in the reservoir was changed to 1.0 N HCl for the following 40—50 ml. The positions of the NA and NM were determined by reading the fluorescence at 335 mμ (excitation wavelength 285 mμ) using an Aminco-Bowman spectro-fluoro-photometer. After determining the radioactivity of aliquots from each fraction in the chromatogram, the activity of duplicates of the effluent corresponding to total acids plus traces of radiochemical impurities and the pooled fractions containing the NA and NM peaks respectively was determined.

As previously mentioned radiochemical impurities in the commercial H DL-NA preparation used were removed by ion exchange chromatography.

Substances used

DL-NA-7-H hydrochloride, New England Nuclear Corp., Specific ac

tivity 7 06—7 60 C/mM After removal of radiochemical impurities as mentioned above, the pure NA was kept in a 2 per cent solution of sodium metabisulphite according to Axelrod and Tomchick (1960) In this medium the tritiated NA proved to be quite stable when stored at about pH 4 and -30°C

Serpasil (Ciba)

Reserpine phosphate lyophilized (used for addition to the homogenization medium)

Results and discussion

1 TURNOVER OF AMINES

Results

The time course of the uptake and elimination of tritiated NA in the rabbit heart and the proportion of the total radioactivity residing in the high speed sediment throughout this period is shown in Fig 11, which represents values obtained from two experimental series A third series showed the same general trend but the figures from this differed due to impurities in the H[3]DL NA

Ion exchange chromatography showed that NA accounted for more than 85 per cent of the total radioactivity in the heart one and four minutes after the NA injection (See Fig 14) Since the values shown in Fig 11 represent the radioactivity in the eluate from aluminium oxide which is free from NM, they may be regarded as a measure of the NA taken up into the

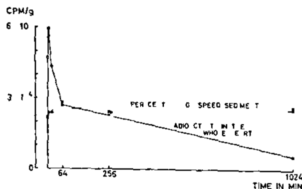


Fig 11 Time course of uptake and elimination of tritiated NA in the rabbit heart and proportion of the total radioactivity found in the high speed sediment

heart, the acid metabolites in the normal heart amounting to less than 10 per cent of the total radioactivity

The elimination of the radioactive NA from the heart occurred at a rate corresponding to a half life for the labeled amine in the heart of about 9 hours, as calculated from the slope of the curve during the interval from 64 to 1024 minutes

The proportion of the radioactive NA found in the high speed sediment was practically unchanged throughout the observation period, in spite of the large variations in total radioactivity in the heart

In control experiments where labeled NA was added to the excised whole heart at 0°C *in vitro* prior to homogenization 95 7 (95 4—96 0) per cent of the radioactivity was recovered from the high speed supernatant whereas 0 4 (0 3—0.5) per cent and 3 9 (3 7—4 1) per cent were found in the high and low speed sediments respectively Thus the distribution of the radioactivity found in the experiments does not represent artefacts due to unspecific binding during the homogenization procedure

Discussion

The rapid uptake and the relatively slow elimination of tritiated NA, found in rabbit hearts in the present experiments is in agreement with previous observations in other species (Whitby et al 1961, Kopin et al 1962 Axelrod et al 1962 Crout 1962 Montanari et al 1963)

According to the *in vitro* findings presented in the previous section of this report the isolated nerve granules accomplish a 50 per cent exchange of their amines with exogenous amines from the incubation medium in less than 10 minutes In view of that it appears significant that steady state in terms of the relative amount of exogenous amines recovered from the high speed sediment in the present *in vivo* experiments was largely established within the first few minutes after the NA injection

The subcellular fractions obtained by the presently available techniques for homogenization and fractionation may be regarded as essentially crude impure and cross-contaminated Even when care was taken to obtain a maximally pure preparation which as a consequence led to decreased amine yield electron microscopy revealed a certain heterogeneity of the sedimented material Gillis 1964 Thus the high speed sediment in the present experiments in addition to the specific amine synthesis and storage granules probably contained mitochondria different kinds of microsomes and possibly "pinched off nerve endings Gray and Whittaker 1960)

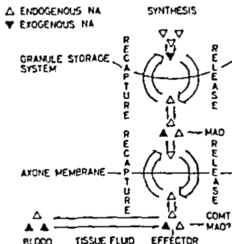


Fig 12 Diagram proposed to represent the dynamics of the recapture mechanism at the axonal membrane and at the level of the intraaxonal granules

The rate of NA release from the granules is assumed to be endogenous and unprovoked of the same order of magnitude in all noradrenergic axones irrespective of what organ they are located in By contrast the rate of release of neurotransmitter from the axone into the surrounding tissue fluid is assumed to be mainly due to the intermittent depolarization of the axonal membrane and is thus probably relatively characteristic for each individual organ At both levels a highly efficient recapture mechanism is assumed to operate preventing undue permanent loss of neurotransmitter

Some of the endogenous locally synthesized NA molecules discharged from the axone will by random diffusion reach the blood stream while similar random diffusion will carry exogenous NA molecules from the blood to the periaxonal area Since the recapture mechanism of the axonal membrane probably is unable to distinguish between endogenous and exogenous CA a certain proportion of the amine molecules recaptured into the axone will be of exogenous origin The degree of "contamination with exogenous amines will be a function of the depolarization frequency the efficiency of the perfusion of the organ and the concentration of exogenous amines in the blood

This model implies that once a NA molecule has been captured by the axone it will be repeatedly discharged from the axone together with endogenous neurotransmitter and recaptured until it is ultimately lost by becoming attached to the trigger mechanism of the effector cell by intra or extra axonal enzymatic destruction or by random diffusion back to the blood stream

However it appears probable that the high speed sediment had a relatively high content of specific amine granules derived from the noradrenergic axones of the tissue This is strongly supported by the finding (Potter and Axelrod 1963b) that a corresponding fraction isolated from the rat heart contained material, which on incubation *in vitro* showed many similarities to the isolated granules from bovine splenic nerves Thus these par

ticles had a high NA and ATP content, giving a molar CA/ATP ratio of about 4:1. They were able to synthesize NA by β-hydroxylation of added DA. Their stability at low pH, to detergents and to hypotonicity was similar to that found in the bovine nerve granules. They showed similar spontaneous release of amines on incubation at 37° C, while they maintained a high NA content at 0° C. They were also shown to take up labeled NA from the incubation medium. A rapidly completed, limited uptake did occur at 0° C, but the uptake at higher temperature was considerably larger and showed a definite time course. On two important points these particles differed from the bovine nerve granules. Thus reserpine addition at the concentrations shown to be highly active in isolated bovine nerve granules was quite inactive in the rat heart particles, both in altering the spontaneous release and in affecting amine uptake. Furthermore, the amine uptake in these particles was not stimulated by addition of ATP-Mg.

In spite of these reported differences, the above mentioned data strongly suggest that the high speed sediment studied in the present investigation contained particles corresponding to the bovine nerve granules used for the studies in the preceding section of this report. Thus one may conclude from the present data that both the noradrenergic axonal membrane and the specific intraaxonal granules possess highly efficient uptake mechanisms whereby they capture amines from their respective surrounding medium, as previously proposed (1, 12).

On the basis of observations of the rapid and specific uptake of labeled circulating CA into the noradrenergic axons the concept of tissue binding rather than enzymatic inactivation as the main mechanism for the termination of the biological action of circulating (Axelrod et al. 1959) or locally released CA (Rosell, Kopin and Axelrod 1963) has been proposed and widely accepted.

However, while the rapid uptake of NA from the extracellular fluid into the noradrenergic axones will lead to termination of its opportunity to stimulate the receptors, the biological significance of this uptake may also be regarded from a different point of view, as an expression of the principle of neurotransmitter economy. The intermittent, randomly spaced NA release from the axones, which apparently occurs even under resting conditions as reflected in the presence of "miniature end plate potentials" in smooth muscle (Burnstock and Holman 1963), or in the resting "overflow" NA leakage into the blood stream (Brown and Gillespie 1957, Rosell et al. 1963), may not impose a severe strain on the NA production in the axones. But the massive discharge of NA resulting from coordinated depolarization of sympathetic [illegible] may well lead to its release in substantial pro-

portion of its total amine content Unless the major portion of the excess NA which does not reach the specific receptors is recaptured into the axones after the membrane has been repolarized, the NA stores in the axones would presumably be rapidly depleted (Paton 1960, Hertting and Axelrod 1961, Hukovic and Muscholl 1962)

The striking rapidity of the uptake into the heart of exogenous NA found in the present investigation demonstrates the high efficiency of the "recapture mechanism However on nerve stimulation at a high frequency the recovery period may be shortened beyond a critical level, and the recapture will then probably become insufficient New "endogenous NA synthesized at a rate of the order of 30—200 ng/g/hour (Spector, Melmon and Sjoerdsma 1962, Spector et al 1963, Montanari et al 1963), may not be able compensate for the continuous loss The result would be a gradual decrease of the total amount of neurotransmitter in the axone This is supported by the observation that intense and long continued sympathetic nerve stimulation caused a considerable reduction of the NA content of skeletal muscle of the cat (Kernell and Sedvall 1964)

On the basis of an investigation of the uptake of circulating NA it has been proposed that a certain proportion of the total amount of the neurotransmitter in each individual organ is not synthesized locally but derived from the blood stream (Kopin and Gordon 1963b) In the rat heart the contribution given by exogenous amines was found to represent about 20 per cent of the total NA

These findings may suggest that the synthesis capacity of the norad renergic axones is insufficient and needs the support by the additional mechanism of uptake of ready made amines from the circulation

However such a concept requires the existence of some inexhaustible and as yet unidentified source which could synthesize and provide the NA to the circulation Moreover it implies that the uptake of circulating NA represents a net increase of the total content of NA in the organ Such net uptake has been shown to occur, temporarily, when large amounts of NA were administered *in vivo* (Raab and Gigee 1955 Mucholl 1960 Wegman and Kako 1961 Campos and Shideman 1962) or in isolated perfused organs (Muscholl 1960 Kopin and Gordon 1962, Iversen 1963) The observation that repletion of various organs in the rat previously dep leted by reserpine treatment, was retarded by demedullation of the adrenal gland (Bhagat and Shideman 1964) indicates that net uptake of circulat ing CA may indeed be a mechanism of importance under special circum stances

However the uptake of NA from the extracellular fluid into the norad

renergic axones under more physiological conditions may well represent randomly occurring exchange of endogenous for exogenous NA. This is supported by the well known fact that the total amount of neurotransmitter in the tissues is normally maintained at a fixed level, characteristic of each individual organ (Euler 1951). In the absence of drug treatment this level was unaffected by adrenalectomy (Euler 1951) or adrenal demedullation (Bhagat and Shideman 1964). The observations that an increase in the organ content of NA occurred after preganglionic denervation (Sedvall 1964), after ganglionic blockade (Brodie and Beaven 1963) or treatment with bretylium (Ryd 1962) or bretylium-like compounds (Costa et al. 1962) indicate that the NA stores in the organs under resting conditions are not quite filled to capacity. Thus a small net uptake into physiological storage sites may occur on infusion of large amounts of NA. By the technique of subcellular fractionation it was demonstrated that the storage capacity of the tissues may be temporarily expanded by accumulation of exogenous amines into a non-particle bound storage form, possibly in the cytoplasm of the noradrenergic axones (Wegmann and Kako 1961, Campos and Shideman 1962). However, the post-infusional elevation in the urinary excretion of CA observed both in man and experimental animals after the termination of a CA infusion indicates that the surplus of CA forced into the axones, by raising its plasma concentration above physiological levels, is relatively rapidly eliminated (Euler 1962).

2. TIME COURSE OF THE EFFECT OF RESERPINE ON THE CONTENT OF NA AND ON THE UPTAKE OF H3-DL-NA IN THE RABBIT HEART

Results

Intravenous injection of reserpine (0.25 mg/kg) caused a profound depletion of the NA content of the rabbit heart, clearly visible 17 minutes after reserpine and almost complete after 1½ hours (Fig. 13). The NA content remained low for about 42 hours and then slowly increased to reach 15–20 per cent of the normal level at 68 hours. Meanwhile the clinical signs of the reserpine treatment, as sedation, ptosis and miosis, had disappeared almost completely.

During the initial phase after the reserpine injection the uptake of labeled NA in the heart was reduced even more strongly than the total NA content. After about 17 hours a gradual recovery of the uptake of exo-

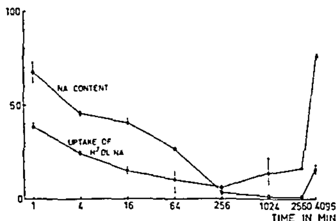


Fig 13 Time course for the effect of reserpine on the NA content and on the uptake of H DL-NA in the rabbit heart *Reserpine 0 25 mg/kg was injected intravenously at 1 to* 4096 minutes before the intravenous injection of H^3DL-NA 82 μC/kg and the animals were killed 16 minutes after the NA administration

Ordinates NA *content and uptake of* H^3DL-NA *in per cent of values in non reserpine* treated controls

Abscissa Time interval between the reserpine and the NA injections The rabbits were killed 16 minutes later

genous NA took place strongly accelerating between 42 and 68 hours after reserpine, i e the time when clinical recovery was observed Thus the signs of clinical recovery were far better correlated to the reestablishment of normal uptake of exogenous NA than to the repletion of the emptied NA stores

Discussion

The time course of the depletion and the starting repletion of NA in the rabbit heart after reserpine administration is in close agreement with previous observations (Carlsson et al 1957a)

While the early fall in the NA content of the heart was quite rapid leading to a 50 per cent depletion in less than twenty minutes the NA remaining at this time disappeared much more slowly as also observed by the abovementioned authors The biphasic course of the curve suggests that the mechanism of the reserpine induced depletion is of a complex nature

Relevant to this problem may be the observation that in many species intact innervation is essential to obtain an optimal amine depleting reserpine effect This has been clearly demonstrated by reserpine administration after denervation of the adrenal medulla in cats (Holzbauer and Vogt

1956) and rabbits (Kroneberg and Schumann 1957, Carlsson et al. 1957a), which resulted in no or at least strongly reduced depletion of the denervated adrenal as compared to the innervated control gland. However, a direct amine releasing effect of reserpine on the adrenal medulla was demonstrated in the rabbit (Kroneberg and Schumann 1958), in the cat (Stjärne and Schapiro 1959) and in the rat (Mirkin 1958, Callingham and Mann 1958, Hillarp 1960b).

Similar observations have been made concerning the reserpine induced depletion of noradrenergically innervated tissues. Thus decentralization by ganglionic blockade or preganglionic denervation reduced or delayed the depletion caused by reserpine in the rat heart (Kirki et al. 1959, Hertting et al. 1962). Decreased or delayed effects of reserpine on the NA content of brown adipose tissue of the rat after postganglionic denervation (Weiner, Perkins and Sidman 1962), or of the rat submaxillary gland after postganglionic denervation or pretreatment with bretylium or guanethidine (Benmiloud and Euler 1963) also suggest that the reserpine effect is partly dependent on intact communication with the central nervous system.

However, decentralization was reported not to interfere with the NA depletion in the nictitating membrane or superior cervical ganglion of the cat (Kirpekar, Cervoni and Furchgott 1962). Nor did sectioning of the spinal cord at the cervical level strongly affect the reserpine induced depletion of the NA in the rabbit heart (Brodie et al. 1957).

There is no evidence that reserpine induces a significantly increased frequency of the nerve impulses in the peripheral sympathetic nerves (Iggo and Vogt 1960). Thus the above mentioned observations suggest that intermittent depolarization may play a permissive role in the reserpine induced depletion of the neurotransmitter stores. This is supported by the finding that the vasoconstrictor response to electrical nerve stimulation in the hind leg of the cat deteriorated much more slowly if the lumbar sympathetic chain was sectioned immediately prior to the reserpine administration (Rosell and Sedvall 1962). Moreover, after a fixed number of electrical nerve stimuli the vasoconstrictor response was completely abolished in the reserpine treated animal, while even prolonged stimulation in the normal animal did not lead to failure of the impulse transmission. The authors discussed the possibility that the intermittent depolarization of the noradrenergic axones occurring even in the absence of signs of increased central sympathetic activity may be the factor responsible for the actual emptying of the NA stores of the axones. These observations were recently confirmed and extended in a study of the time course of the reserpine induced depletion of the NA stores in the innervated and acutely denervated skeletal

muscle of the cat (Sedvall and Thorsson 1963) Unilateral sympathetic denervation prior to the reserpine administration resulted in the preservation of about 20 per cent of the original NA content on the denervated side, at a time when the innervated side was completely depleted The NA remaining proved to be resistant to further reserpine injections while it was readily released by electrical nerve stimulation producing vasoconstriction

Thus the biphasic course of the NA depletion of the rabbit heart observed in the present experiments may well be due to differences in the time course of the development of the central and peripheral effects of reserpine However the biphasic course may also express a local action of reserpine at two different levels in the organ the axonal membrane and the intraaxonal granules This problem will be dealt with in the following section

In the present investigation the depression of the uptake of circulating NA into the heart preceded the progressive decrease in its total NA content The reestablishment of normal uptake capacity also occurred much earlier than the return to a normal total NA content in the organ at between 42 and 68 hours after reserpine thus showing a much closer correlation to the clinical recovery which was completed at about the same time in agreement with the observations of Andén et al (1964) in reserpine treated rats

These findings imply that normal function in the peripheral noradrenergic system is possible in the presence of a definitely sub-normal total NA content in the tissue Lundborg 1963 Carlsson et al 1963b Andén et al 1964 Carlsson 1964) On the basis of evidence obtained in experiments with reserpine as well as other drugs α methyl meta tyrosine metaraminol and D-A (Andén and Magnusson 1963) it was concluded that normal impulse transmission in the peripheral noradrenergic system requires intact functioning of a mechanism taking up NA into an intra axonal particulate fraction from which the release of the neurotransmitter was postulated to occur Carlsson 1964)

The present experimental results confirm the observations mentioned above that neurotransmission in the peripheral noradrenergic system of the rabbit lost after reserpine treatment, appears to be restored simultaneously with the return of normal functioning of the mechanisms responsible for uptake and retention of circulating NA However they do not provide evidence for the identity of the fraction from which the neurotransmitter was released on depolarization of the axonal membrane

As briefly reviewed in the introduction to this report there is consider-

Discussion

The present experiments clearly demonstrate a reduced uptake and/or retention of circulating NA in the hearts of reserpine treated rabbits. At 1 and 4 minutes after an intravenous injection of tracer amounts of H^3DL-NA, the amount of radioactive material retained in the hearts of rabbits pretreated 64 minutes earlier with a *moderate intravenous reserpine dose*, was less than one half of that found in the hearts of normal rabbits.

The simultaneous increase in the relative and absolute amount of acid metabolites found in the hearts from reserpine treated rabbits indicates that the decrease in the total radioactivity may not be exclusively due to inhibition of passage of the exogenous amines across the axonal membrane into the axoplasm, since deamination of CA is assumed to largely occur intra axonally (Spector et al 1960). The increased rate of deamination is compatible with the concept that the reduction of the retention capacity for circulating NA is due to reserpine induced inhibition of uptake of the newly arrived amines into the specific intraaxonal granules, and consequent increased exposure to enzymatic inactivation by intra axonal monoamine oxidase (Carlsson 1964). However, the net increase in deaminated metabolites at 1 minute corresponded to only 13 9 per cent, and at 4 minutes to 5 2 per cent of the deficit in the total amount of radioactivity retained in the hearts of reserpine treated rabbits as compared to untreated controls. Thus the present results can be explained by selective inhibition of uptake into the intraaxonal granules in the absence of impairment of amine passage across the axonal membrane into the interior of the axone only if it is assumed that the large deficit in total radioactivity corresponds to deaminated material, which at 1 minute after the NA injection had already diffused out of the tissue. However, during the time interval between 1 and 4 minutes after the NA injection no further reduction was seen in the labeled NA content of the hearts from reserpine treated rabbits as compared to non treated controls.

Thus the labeled NA found in the heart at 1 minute after the NA injection was apparently stored by a mechanism which protected it against destruction by mono-amine oxidase. This suggests that even in the reserpine treated rabbits the uptake of exogenous NA into the specific intraaxonal granules after passage through the membrane of the axone was *largely completed in less than 1 minute. Thus amine uptake into the granule* fraction was at least not completely inhibited at this time and under the experimental conditions used. Since apparently little further deamination

took place between 1 and 4 minutes after the NA injection, the remaining excess acid metabolites found in the hearts of reserpine treated rabbits at 4 minutes must be assumed to represent material formed during the first minute Thus the rate of diffusion of the deaminated metabolites out of the axone did not appear to be very high

In view of this evidence the marked reduction in the uptake and retention of circulating amines in the hearts of reserpine treated rabbits appears to be difficult to explain in terms of an exclusive effect on the amine uptake into the intraaxonal granules It may be justified to postulate that the effects of reserpine during the early rapid phase of the depletion were exerted at more than one level in the noradrenergic axone

The evidence in the literature concerning the nature of the effect of reserpine on the uptake of circulating amines is rather conflicting Two basic facts may be of value to reconcile apparent discrepancies

Firstly, there is considerable evidence that reserpine inhibits monoamine uptake in isolated platelets by interference with specific amine transport mechanisms (Brodie et al 1957, Hughes, Shore and Brodie 1958, Hughes and Brodie 1959) This has been shown to apply to CA uptake in isolated adrenal medullary granules as well (Kirshner 1962) The inhibition of amine transport in these granules (Carlsson et al 1963a Jonasson et al 1964) as well as in isolated nerve granules (the present investigation) has been shown to be competitive Thus it was possible to overcome the reserpine induced inhibition of amine uptake into the granules by raising the amine concentration in the external medium

Moreover, as previously mentioned the inhibitory effect of reserpine on amine uptake has a definite time course Under the present experimental conditions beginning recovery of the amine uptake capacity was seen at about 17 hours after the reserpine injection

Thus the reserpine dose and administration route used the time interval from the reserpine injection to the CA administration the resulting extra cellular concentration of CA, as well as possible organ and species differences must be kept in mind in considering the results in the literature in this field

The first evidence of exogenous NA uptake in the noradrenergic axones in reserpine treated animals, was purely functional Thus administration of NA led to temporary functional recovery of the response to TA or electrical nerve stimulation or both (Burn and Rand 1958 Gillespie and Mackenna 1959 1961, Rosell and Sedvall 1961) The latter authors who studied the vasoconstrictor response in the skeletal muscle of the cat hind leg noticed that a NA infusion at 20—24 hours after the reserpine ad-

ministration was consistently effective in restoring the vasoconstrictor response, while similar infusion at 5 hours after reserpine failed to do so

The uptake of circulating amines into the tissues was reported to be strongly reduced by reserpine treatment (Muscholl 1960, Hertting et al 1961 Crout et al 1962, Andén et al 1963 1964) However an apparently normal uptake even after reserpine pretreatment was found in the kidney and uterus of the pithed cat on administration of large amounts of NA (Pennefather and Rand 1960), and in the heart and spleen of the pithed rat also with the use of relatively high plasma concentrations of CA (Weiner and Trendelenburg 1962) A definite, but subnormal amine uptake was found in the heart of the reserpine-treated dog (Campos and Shideman 1962 Gaffney, Morrow and Chidsey 1962) and in the isolated perfused heart of the rat pretreated with reserpine (Kopin et al 1962)

Thus a certain amine uptake apparently does occur in many tissues even after adequate reserpine treatment By subcellular fractionation technique the major portion of the exogenous amines taken up into the heart of the reserpine treated dog was found to be stored in non particle bound form (Campos and Shideman 1962) This is in agreement with recent morphological evidence Thus the exogenous NA taken up into the noradrenergic axones of the iris of the reserpine treated rat on administration of large amounts of NA did not show the distribution of the fluorescent material characteristic of normal animals Instead of being concentrated to the "varicosities along the axones it occurred diffusely in the axoplasm The restoration of the fluorescence after NA administration was short lasting but was considerably prolonged by pretreatment with monoamine oxidase inhibitors (Hamberger et al 1964)

The above mentioned data strongly suggest that amine uptake into the specific intraaxonal granule fraction may be inhibited by reserpine However several problems concerning the mechanism of the early effects of reserpine appear to be difficult to solve by assuming that the drug acts exclusively by inhibiting amine uptake at the granule level

Thus according to the only direct *in vitro* evidence concerning isolated "heart granules so far available reserpine at concentrations below 10⁻⁴M neither changed the spontaneous release nor the uptake of exogenous amines from the incubation medium Potter and Axelrod 1963b) In view of the well documented effects of reserpine on isolated granules from bovine splenic nerves the possibility that the failure of reserpine to produce its expected effects in this preparation may be due to technical difficulties must be kept in mind This is further supported by the observation that *in vivo* pretreatment with reserpine reduced but did not completely abolish exo-

genous amine uptake *in vitro* in the corresponding subcellular fraction isolated from the rat brain (Mirkin Giarman and Freedman 1963)

Assuming that the results obtained with the granule fraction isolated from bovine splenic nerves may be applied to *in vivo* physiology it may in this connection be relevant to point out that reserpine addition considerably delays but does not completely inhibit the spontaneous amine release *in vitro* (Euler and Lishajko 1963b) At 37°C addition of reserpine at low concentrations prolonged the time required for a 50 per cent depletion of the nerve granules from 10 to about 60 minutes Thus if a 50 per cent NA depletion occurs *in vivo* in shorter time as a result of treatment with reserpine the conclusion must be that the NA loss was restricted to non granule bound NA (Weil Malherbe and Bone 1959 Johnson 1964), or that the depletion had occurred before reserpine had penetrated into the axoplasm in sufficient concentration to affect either uptake or release processes in the granules As previously pointed out a 50 per cent depletion of the NA in the rabbit heart was in the present experiments accomplished in less than 20 minutes while a similar reduction in the amine uptake capacity was established even earlier This suggests that the inhibition of uptake may have been exerted at a level different from that of the intraaxonal granules since inhibition of amine uptake in adrenal medullary granules according to the studies previously referred to showed a latency before the uptake capacity was reduced to one half, of the order of 6 hours (Bertler et al 1961 Lundborg 1963 Carlsson et al 1963b)

On the basis of tissue slice experiments where amine uptake was shown to be inhibited by the same low concentrations of ouabaine or reserpine, which had been shown to inhibit amine transport into blood platelets, the reduced amine uptake in the presence of reserpine was proposed to be due to inhibition of the specific amine transport mechanism of the axonal membrane (Dengler et al 1962) This is further supported by the observations that the strong reduction of uptake of H^3DL-NA into slices of mouse cortex caused by addition of reserpine to the medium at a 5×10^{-6}M concentration was not affected by preincubation with mono-amine oxidase inhibitors (Ross 1964) The same author observed that reserpine when added to slices which had previously been allowed to accumulate tritiated NA accelerated the spontaneous release of the labeled amine contrary to the results obtained with isolated bovine splenic nerve granules These results also strongly suggest that the effect of reserpine is not exclusively restricted to the granules, but occurs at some additional level possibly the transport system at the axonal membrane

The rate of disappearance of the CA from the tissues after reserpine ad

ministration was found to occur at a rate characteristic for each individual organ (Carlsson et al 1957a), the rate of depletion showing a clearcut correlation to the amine turnover rate of the organ (Bertler 1961) As previously pointed out the nerve granules apparently constantly release and recapture amines at a rate, which is presumably "endogenous , of the same order of magnitude in the granules in all noradrenergic axones and thus independent of the state of activity of the axone The major portion of the neurotransmitter intermittently released from the axone on depolarization of the axonal membrane, is also presumably recaptured by the highly efficient uptake mechanism at the axonal membrane The frequency of these depolarizations is not uniform in all noradrenergic axones but probably under resting conditions relatively characteristic for each organ The nerve impulse frequency as well as the efficiency of the perfusion of the organ are presumably the factors determining the amine "turnover , defined as the rate of disappearance from a noradrenergically innervated organ of a population of individual neurotransmitter molecules

In view of this the abovementioned observation that reserpine depletes the organs at a rate depending on the "turnover rate of their amines appears to support the proposition that this drug exerts its early rapidly depleting effects on organs like the heart by primarily interfering with the amine recapture mechanism of the axonal membrane rather than that of the intra axonal granules

Conversely in organs having a slow amine "turnover rate this early reserpine effect may be of minor importance while the gradually developing inhibition of the granule transport mechanism may in these cases dominate the picture

Conclusions

Equilibration of tritiated NA taken up into the noradrenergic axones between the particle bound and "free NA pools was nearly completed during the first minute after the NA injection This confirms the *in vitro* observations of rapid amine uptake into the isolated bovine splenic nerve granules

The much more rapid rate of *appearance* of labeled amines in the heart than of their subsequent *disappearance* supports the concept that a highly efficient recapture mechanism presumably located at the level of the axonal membrane takes up a major portion of the neurotransmitter intermittently discharged from the axone thus subserving the principle of neurotransmitter economy

The biphasic course of the depletion of the rabbit heart after an intravenous injection of a moderate reserpine dose may be due to differences in the latency for the central and peripheral effects of the drug Alternatively, it may indicate that the local effects of reserpine are exerted at more than one level in the noradrenergic axone

The depression of the uptake of circulating labeled NA into the heart clearly preceded the decrease in its total NA content The restoration of normal uptake also occurred earlier than that of a normal NA content, and thus showed a much better correlation to clinical recovery

Since observations in the literature indicate that neurotransmitter release on electrical nerve stimulation occurs from a reserpine resistant NA pool, the present results were interpreted as indicating that recovery of normal impulse transmission depends on restoration of normal uptake capacity in a NA pool, different from the specific intraaxonal granules and possibly related to the axonal membrane

In rabbits killed 1 or 4 minutes after an intravenous injection of tritiated NA the total amount of radioactive material recovered from the hearts of animals pretreated with reserpine about one hour earlier was less than one half of that found in non treated controls Chromatographic analysis showed that the radioactivity recovered as NA was reduced to less than one third of the control value while the recovery as normetanephrine was unchanged or subnormal and the recovery as "acids strongly increased above the control levels These findings were interpreted as indicating a dual local action of reserpine inhibition of recapture of amines at the level of the axonal membrane dominating the early rapid release phase in organs like the heart, while the gradually increasing degree of inhibition of amine uptake into the intracellular granules may dominate the picture in organs more slowly depleted by reserpine, as for instance the adrenal medulla

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was weighed, multiple superficial incisions, about 0.5 mm deep, were made at random into the capsule and the main vein was cannulated.

The perfusion medium (Tyrode's solution ($NaCl$ 1.37×10^{-1} M, KCl 2.68×10^{-3} M, $CaCl_2$ 1.8×10^{-3} M, $MgCl_2$ [illegible] 10^{-3} M, $NaHCO_3$ 1.19×10^{-2} M, NaH_2PO_4 3.6×10^{-4} M, glucose 5.5×10^{-3} M) aerated with 5 per cent carbon dioxide in oxygen, was maintained at 37°C and pumped into the organ using a constant flow pump (Sigmamotor) giving pulsatile flow and adjusted to give a minute volume of 3 ml. The effluent from the scarifications was collected into series of ice cooled graduated glass cylinders.

The amine secretion from the gland was stimulated by repeated injections at 1½ minute intervals of 5 μg of acetylcholine in constant volumes of 0.05 ml via a polyethylene catheter, the tip of which projected to a point close to the cannula tied into the vein constituting the entrance to the organ. Physostigmine 1 μg/ml was added to the perfusion medium.

Extractions

All steps during the extraction were carried out at 0 – 2°C. After the perfusion was finished the perfusate was centrifuged at 2500 × g for 10 minutes to remove the small amounts of blood cells expelled from the organ. The supernatant was decanted and deproteinized by addition of 1/10 volume of 4 N perchloric acid. The protein precipitate was removed by centrifugation and the clear supernatant used for CA and purine material analysis.

The perfused gland and the control organ, which was kept at 0°C during the perfusion, were both homogenized by grinding with quartz sand in a porcelain mortar and extracted with 10 volumes of 0.4 M perchloric acid. After centrifugation for 10 minutes at 3000 × g the supernatant was decanted and the sediment extracted twice with 0.2 M perchloric acid. The pooled supernatants were brought to pH 5–6 with potassium hydroxide. After removal of the perchlorate precipitate by centrifugation the clear extract was stored at –30°C.

The acid insoluble sediment was hydrolyzed by heating to 100°C for 1 hour in 70% perchloric acid (Marshak and Vogel 1951). The hydrolysate was neutralized with potassium hydroxide and centrifuged to remove the perchlorate precipitate. After freezing, thawing and recentrifugation the clear extract was kept at –30°C until analysed by ion exchange chromatography.

Density gradient centrifugation

A sucrose density gradient was prepared according to Hillarp (1958c). In 3 ml nylon tubes (MSE swing-out head), 0.5 ml of 1.5 M sucrose was layered on top of 0.5 ml of 1.8 M sucrose.

The perfused medulla was carefully separated from the cortex in the cold and squeezed between nylon rollers according to Euler (1958). The press juice thus obtained was diluted with 0.3 M sucrose and centrifuged at about 900×g for 10 minutes. The supernatant was then recentrifuged at 30 000×g for 30 minutes. The second supernatant was decanted and the loose layer on top of the sediment removed by swirling with sucrose. Then the sediment was resuspended in 0.3 M sucrose and recentrifuged. After decanting and repeating the swirling with sucrose to remove the small amounts of loose layer remaining, the sediment was resuspended in 4 ml of 0.3 M sucrose.

One ml of the resuspended sediment was carefully layered on top of the sucrose gradient and the tube was left in the refrigerator at about +2°C overnight. The next day it was centrifuged at 50 000×g for 60 minutes. The upper layers were decanted and the bottom "heavy granule" sediment was washed and extracted with 0.4 M perchloric acid. The extract was allowed to stand in ice water for about 10 minutes. Then it was diluted and centrifuged at 50 000×g for 30 minutes. The supernatant was decanted and brought pH 6—7 with potassium hydroxide. The extract was frozen and thawed out and the perchlorate was removed by centrifugation. The clear extract was then ready for chromatographic analysis.

Assay methods

The CA content of the extracts was determined by the fluorimetric technique of Euler and Lishajko (1961b).

ATP was determined by a modification of the luciferase method of Strehler and Totter (1953) as previously described.

Control experiments showed that the CA and ATP content of the two adrenal glands from the same animal, calculated per unit weight, were nearly equal in accordance with previous observations concerning the CA (Elliott 1912, Butterworth and Mann 1956, Draskoczy et al. 1963).

Because of the high ionic strength of the perfusate the purine material in this was absorbed on a column (0.5×20 mm) of Norit, eluted with 40

ml of ethanol water ammonia 100 100 1 (Hurlbert 1957) and lyophilized prior to analysis by ion exchange chromatography

Anion exchange chromatography

The neutral extract was passed through an anion exchange column (Dowex 2, 200—400 mesh 150×4 mm formate form) The elution (2 5 ml fractions flow about 7 5 ml/hour) was performed using the gradient method of Hurlbert et al (1954) The eluant in the reservoir consisted of 4 M formic acid, 1 M ammonium formate The mixer volume was 250 ml The elution pattern was followed by reading the ultraviolet absorption at 260 mm, using a Beckman DU instrument

Cation exchange chromatography

The purine and nucleoside content of the extract was analysed using the strong cation exchange resin, Dowex 50 X 4 (200—400 mesh hydrogen form), with the same column and flow rate Gradient elution was used beginning with 1 N hydrochloric acid in the reservoir and after about 60 fractions shifting to 2 N hydrochloric acid The mixer volume was 250 ml

The identity of the different peaks was tentatively determined by reading their ultraviolet absorption spectra and comparing this and their elution position on ion exchange chromatography with the same data for known substances

The chromatography was carried out at a constant temperature of 0°—2 C

Radioactivity determination

Radioactivity was measured in a Tri Carb Liquid Scintillation Spectrometer, as previously described

Substance used

Adenine 8 C^{14} 8 5 m C/mM (California Corp Biochem Res)

Results

The CA in the perfusate consisted to 47.8 (44—51.5) per cent of NA. The output from the perfused gland was very high during the first collection periods and then decreased with time (Fig. 16). Repeated injections of acetylcholine in the presence of physostigmine resulted in an approximate doubling of both the A and NA output.

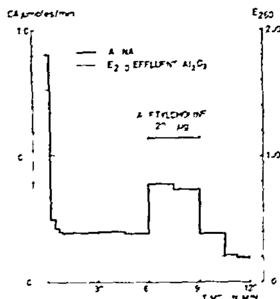


Fig. 16. Adrenal perfusion experiment. CA release and content of material in the perfusate showing UV absorption at 260 mµ (after passing the perfusate through aluminium oxide to remove the CA). Secretory effect of acetylcholine.

Table IV. [illegible]

	NA µmoles	A µmoles	CA µmoles	NA per cent	ATP µmoles
Before perfusion	4[illegible]	99.5	13[illegible]	34	33.0
After perfusion	4	3.5	118[illegible]	0.1	[illegible]
Deficit	22.[illegible]	-4.0	18.5		[illegible]3
Found in perfusate	10.0	1[illegible]	22.7	44.0	
Net gain [illegible]	1[illegible]	1[illegible]	4.2		

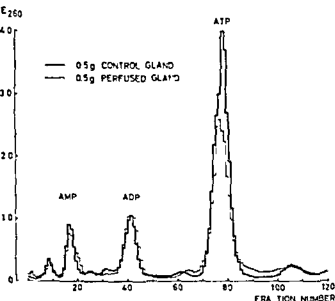


Fig 17 Anion exchange chromatograms (Dowex 2) of 0 5 g of control and perfused medulla

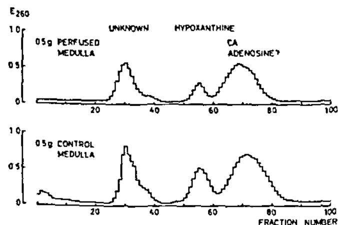


Fig 18 Cation exchange chromatograms (Dowex 50) of 0 5 g of control and perfused medulla

NA made up 36 (34 5—37 5) per cent of the total CA in the control gland In the perfused gland the relative NA content was reduced to 20 6 (20 8—30 3) per cent Thus more NA than A was lost during the perfusion Table IV shows a typical experiment The NA content of the perfused gland was reduced to about one half of the starting level, whereas the A content was unchanged or even slightly increased in comparison with the control gland Thus the A released into the perfusate was quantitatively replaced by synthesis i e methylation of noradrenaline The NA lost was possibly also to ome extent compensated for by synthesis

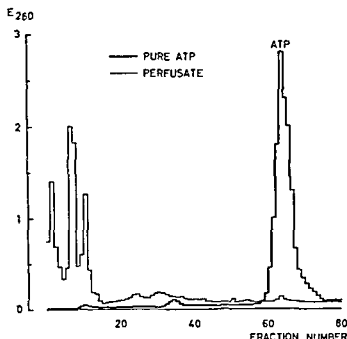


Fig 19 Anion exchange chromatograms (Dowex 2) of aliquots of perfusate and of pure ATP after adsorption on Norit and elution by the same procedure as that used for the perfusate. No ATP, ADP or AMP was found in the perfusate. The third peak in the chromatogram of the perfusate eluted at the position characteristic of AMP had a different spectrum showing a maximum extinction at 280 mμ

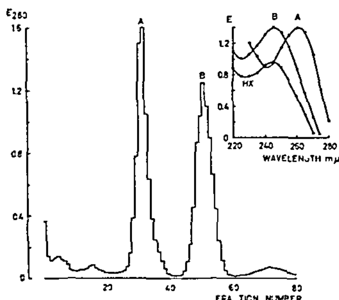


Fig 20 Cation exchange chromatogram (Dowex 50) of aliquot of the perfusate and ultraviolet absorption spectra of peaks A and B and of pure hypoxanthine (HX). Peak A had an adenine like spectrum while peak B showed both the same elution position and ultraviolet absorption spectrum as pure hypoxanthine

The ATP content of the perfused gland was reduced with 22 per cent in comparison with the control gland while the ADP and AMP were unchanged (Fig 17). The bases of the perfused gland were also slightly reduced (Fig 18)

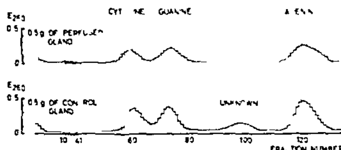


Fig 21 Cation-exchange chromatogram (Dowex 50) of the acid hydrolysate of acid insoluble material from 0 5 g of a control and a perfused adrenal gland respectively The bases were tentatively identified by determining their UV absorption spectra in acid and alkaline media Uracil was not adsorbed on the column and was thus recovered in the effluent

In the experiment in Table IV a total of 22 7 μmoles of CA were found in the perfusate However, no ATP could be demonstrated in this, by anion-exchange chromatography (Fig 19) Nor did the perfusate contain any ADP or AMP In cation exchange chromatograms of the perfusate two major peaks were found having absorption spectra characteristic of purine compounds (Fig 20) The identity of the second peak with hypoxanthine was checked by comparing its absorption spectrum in the ultraviolet and its elution position with the corresponding data for the pure substance The first peak, which turned out to be compound, has not yet been identified

Cation-exchange chromatography of the acid hydrolysate of the acid insoluble sediment obtained on perchloric acid extraction of the glands revealed a uniform decrease in the total amount of RNA bases in the perfused gland both in the cortex and in the medulla (Fig 21)

The turnover rate of the different adenine related compounds in the gland was studied by infusing radioactively labeled adenine into the gland and following its appearance in the different purine nucleoside and nucleotide fractions of the perfusate as well as of the medulla and cortex of the perfused organ by anion and cation exchange chromatography

In extracts of whole perfused medulla a considerable degree of incorporation of labeled adenine was found in AMP, ADP and ATP (Fig 22) The specific activity (SA) of the small amount of ATP found in the cortex was even higher (Fig 23) A considerable amount of radioactive material was found in the bases both from the medulla (Fig 24) and from the cortex while no radioactive adenine incorporation was visible in the adenosine

terial, in the form of ATP or any of its break down products appears in the effluent from the adrenal gland concomitantly with amine relase

Moreover the presence of active amine and nucleotide synthesis, the clearcut response in amine secretion to acetylcholine stimulation, potentiated in the presence of physostigmine, the inhibitory effect of higher doses of acetylcholine and the relatively intact nucleotide pattern of the perfused gland, all indicate, that although the organ was undoubtedly severely damaged it still retained some degree of biochemical integrity, enabling it to respond "physiologically" to specific stimuli

In the present experiments the ATP lost from the adrenal medulla did not appear as such in the effluent This does not allow definite conclusions concerning the fate of the ATP lost from the medulla *in vivo*, but it gives no support to the concept of release of the intact ATP molecule from the medulla concomitantly with amine secretion (Hillarp 1958d)

However, the perfusate contained large amounts of hypoxanthine and additional, as yet unidentified compounds which showed considerable radioactivity on infusion of labeled adenine and one of which had an adenine like spectrum but a different elution position on chromatography

The medullary storage granules themselves posses the enzymes necessary for the dephosphorylation of ATP to AMP (Hillarp 1958a) Moreover, the adrenal medullary cells have been shown to be able to deaminate the adenine compounds, ultimately leading to the formation and accumulation of hypoxanthine (Hillarp 1960a)

Thus the hypoxanthine found in the perfusate as well as the unknown compound having adenine like spectral characteristics may theoretically be derived from the ATP of the medullary granules

On infusion of labeled adenine a considerable radioactivity was found in all purine and nucleotide fractions of the perfused gland The SA of the compounds isolated from the cortex was consistently higher than the SA of those from the medulla In the perfusate the SA of the bases was considerable The nucleotides and the bases from the "heavy granules isolated from the perfused medulla by density gradient centrifugation, showed no or negligible radioactivity

Thus one may conclude that there were no signs of active nucleotide synthesis from adenine in the fraction containing exclusively specific amine granules with negligible contamination of mitochondria or microsomes This is in accordance with the observations of Prusoff et al (1961), who studied the turnover of the terminal phosphates of the ATP in the mitochondria and "heavy granules isolated from the adrenal glands of the rat,

cat and dog by following the *in vivo* incorporation of radioactive phosphorus

During recent years the CA and ATP release from isolated bovine adrenal medullary granules spontaneously occurring on incubation *in vitro*, has been reported to be paralleled by a decrease in their ribonucleic acid content (Philippu and Schumann 1963 a, b) It was also shown that incubation of the granules with ribonuclease led to a dose dependent CA and ATP release The absence of protein loss showed that the ribonuclease effect was not due to unspecific damage to the granules The conclusion of the authors was that ribonucleic acid may be intimately connected with CA storage and that intragranular depolymerization of ribonucleic acid by endogenous ribonuclease may be the factor triggering amine and ATP release The nearly complete depletion of bovine adrenal medullary granule CA and ATP resulting from incubation *in vitro* in the presence of phenoxybenzamine was also accompanied by a strong reduction in their ribonucleic acid content (Stjärne, to be published)

In the present experiments the perfusion resulted in a considerable reduction of the ribonucleic acid fractions of both cortex and medulla, as judged from analysis of the bases from acid hydrolysates of the acid insoluble sediments obtained on perchloric acid extraction No incorporation of radioactive adenine was seen Thus with this technique there were no signs of resynthesis of the ribonucleic acid fraction The gradual loss of ribonucleic acid observed in the present series may possibly be related to physiological amine relase (Philippu and Schumann 1963b) However, it may also represent an experimental artefact expressing the degree of unspecific damage to the organ under the experimental conditions used This loss may possibly be related to the basic changes in the organ causing the abnormal spontaneous CA release and the progressively developing decrease in the secretory response to acetylcholine

Conclusions

The ATP lost from the isolated perfused bovine adrenal gland concomitantly with CA release, was not recovered as such in the effluent from the gland Large amounts of purine bases were found in this

On infusion of radioactively labeled adenine little or no radioactivity was found in the nucleotides from the "heavy granules of the perfused medulla isolated by density gradient centrifugation This shows that the ATP of the granules is metabolically relatively inert, and is thus compatible with

the view that its physiological function may be to serve as anionic component in a CA adenine nucleotide storage complex

CA release from the isolated perfused bovine adrenal gland was accompanied by a decrease in its ribonucleic acid content, similar to that occurring on incubation of the isolated CA storage granules *in vitro* No incorporation of radioactive adenine occurred in this fraction The present data do not allow any definite conclusions concerning the possible physiological significance of ribonucleic acid loss, concomitantly with CA secretion

SUMMARY

Problems concerning the uptake, storage and release of CA, and with the modification of these processes by drugs, notably reserpine, were dealt with.

Experiments *in vitro* with isolated granules from bovine splenic nerves and adrenal medulla confirmed previous observations concerning the similarities between the two types of granule in resting CA/ATP ratio, permeability of the granule membrane to CA, exchange of amines with the incubation medium and in the inhibitory effect on amine uptake of various drugs.

However, important quantitative and possibly qualitative differences between the nerve and adrenal medullary granules were found in stability to hypotonic media, degree of linkage of CA and ATP release spontaneously or as the result of drug addition, and in amine turnover rate.

While the results were regarded as compatible with the concept of storage of part of the CA in the two types of granule in a CA-adenine nucleotide-water soluble protein complex, they also strongly suggested the existence of other mechanisms for binding of amines in the granules. Some of the quantitative and apparent qualitative differences between the nerve and adrenal medullary granules observed in the present experiments were assumed to be due to different proportions in the two types of granule of water soluble amines bound to adenine nucleotides and protein, and functionally water insoluble amines, probably bound to lipid or lipoprotein material in the granules.

The implications of the results of the *in vitro* experiments were expressed by a "granule model", intended to represent a working hypothesis to interpret the experimental data.

Equilibration of tritiated NA taken up into the rabbit heart between the particle bound and "free" NA pools, was nearly completed during the first minute after NA injection. This was regarded as confirmation of the *in vivo* significance of the rapid amine turnover rate observed in the nerve granules *in vitro*. The much more rapid rate of *appearance* of labeled amines in the heart than of their subsequent *disappearance* suggested the existence of a highly efficient recapture mechanism in the axonal membrane taking up a major portion of the neurotransmitter intermittently discharged from the axone, thus subserving the principle of neurotransmitter economy.

The effects of reserpine, both on the total NA content of the rabbit heart and on the uptake into the heart of labeled amines, indicated that the local action of this drug is exerted at more than one level in the axone The difference in the time course of the NA depletion induced by reserpine in different organs reported to be correlated to their amine turnover rate was considered as additional evidence for a dual local action of the drug It was concluded that an inhibitory effect on amine transport mechanisms at the axonal membrane may be mainly responsible for the rapid phase of the depletion in organs like the heart, while the more slowly developing inhibition of the amine transport system of the granules may dominate in organs less rapidly depleted by reserpine as for instance the adrenal medulla

The ATP lost from the adrenal medulla, concomitantly with CA release, was not recovered as such in the perfusate from the bovine adrenal gland, while large amounts of purine bases were found in this No or negligible incorporation of labeled adenine infused into the organ was seen in the nucleotides from the specific amine granules isolated from the perfused medulla by density gradient centrifugation This was regarded as evidence that the ATP of the amine granules is metabolically relatively inert, and was thus considered compatible with the view that its physiological function is to serve as anionic component in a CA adenine nucleotide storage complex.

CA release from the perfused bovine adrenal gland was accompanied by loss of ribonucleic acid material from the organ. No incorporation of labeled adenine occurred in this fraction The present data did not allow any definite conclusions concerning the possible physiological significance of ribonucleic acid loss concomitantly with CA secretion

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EFFECTS OF CARDIOVASCULAR REFLEXES ON NET CAPILLARY FLUID TRANSFER

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CHAPTER I

Introduction

a General considerations

The maintenance of cardiovascular homeostasis implying an appropriate blood supply to the tissues presupposes that the heart constantly receives an adequate return of blood from the tissues for its filling The fundamental factor governing the venous return and the filling of the heart is according to GUYTON the prevailing mean circulatory pressure (GUYTON POLIZO and ARMSTRONG 1954 GUYTON LINDSEY and KAUFMANN 1955 GUYTON 1963 *a*) The preservation of this filling pressure at an optimal level necessitates a proper relation between the *holding capacity* of the vascular system and its *blood content* (cf GUYTON 1963 *b* p 194) If the capacity of the system should increase and/or its blood content decrease it is obvious that the mean circulatory pressure and the venous return to the heart would fall and hence also the cardiac output and the blood supply to the tissues

Disturbances of the normal balance between vascular capacity and blood content can be compensated for principally by adjustments of either one of these two components Alterations in vascular capacity brought about by reflex shifts in venous tone are undoubtedly a very important mechanism for readjusting mean circulatory pressure and venous return as for example following a sudden change in blood volume mainly because it can be so rapidly effectuated It is however well known that this mechanism is not powerful enough to compensate fully for a substantial blood loss The fact that plasma volume returns towards normal soon after a hemorrhage suggests the existence of more slowly acting but equally important mechanisms designed to maintain a constant volume within the cardiovascular system Exactly how these mechanisms operate is as yet not fully understood but one can expect that a multiplicity of processes are directly or indirectly involved in this complex function Any of these processes which can contribute to a reasonably *rapid* restoration of circulating fluid volume must be considered as possibly an important factor for restoring normal cardiovascular function This is especially so if it is sensitively regulated by appropriate feed back systems

From a theoretical point of view two fairly rapid basically different mechanisms seem to be feasible First fluid can be mobilized from the extra

vascular space This implies that fluid depots already available within the organism are utilized for refilling the intravascular compartment Second reductions in the continuous losses of fluid and salt *via* the kidneys can occur eventually combined with an urge for additional fluid intake This means that the fluid exchange between the organism and its external environment can be altered in the direction favourable to the intravascular compartment This latter process which seems to be at least partly governed by cardio vascular receptors has been recently discussed at length (GAUER and HENRY 1963) and will therefore not be dealt with here How fluid mobilization from the extravascular space across the capillary membranes is initiated is but poorly understood and little is known as to whether this process is in any way more directly governed by appropriate reflex mechanisms The present study was undertaken in an attempt to elucidate, in more detail the part that cardiovascular homeostatic reflexes play in the mobilization of fluid from the extravascular space

The principal factors regulating the exchange of fluid across the capillary walls were proposed by STARLING (1896) The rate and direction of the trans capillary flow according to his hypothesis is determined by the relation between the hydrostatic and the colloid osmotic pressure gradients across the capillary walls and by the functional properties of the capillary membrane The trans capillary hydrostatic pressure gradient (mean capillary hydrostatic pressure minus tissue pressure) tends to filter fluid from the blood The colloid osmotic pressure gradient accomplished by the relative impermeability of the capillary wall to plasma proteins will bring fluid back into the blood whenever it ex ceeds the hydrostatic pressure gradient Under normal conditions there is a balance between the hydrostatic and osmotic forces so that no significant net fluid transfer occurs Whenever this normal equilibrium is upset as for example with a change in mean capillary hydrostatic pressure a net trans capillary movement of fluid will take place

The validity of the Starling hypothesis was given substantial experi mental support by the studies of LANDIS (1927) The most important entities upon which the hypothesis is founded *i e* the plasma colloid osmotic pressure and the capillary hydrostatic pressure were estimated directly in LANDIS experiments and could be related to the measured rate of transcapillary net fluid exchange In keeping with Starling s hypothesis it was found that the rate of transcapillary exchange was proportional to the difference between capillary hydrostatic pressure and plasma colloid osmotic pressure Similar confirmatory results were obtained by PAPPENHEIMER and SOTO RIVERA (1948) in their studies of capillary transfer in the leg of cats and dogs using another method for estimation of mean capillary hydrostatic pressure

The principal factors which according to Starling should be responsible for the net fluid exchange across the capillary membranes have thus been experimentally substantiated and his hypothesis is generally accepted (cf Landis and Pappenheimer 1963) On the other hand little is known whether any of these factors can be influenced by suitable control mechanisms so that there would be a true regulation of the fluid partition between the intra and extravascular spaces with the primary aim of maintaining a constant blood volume It seems reasonable that neither tissue pressure nor plasma and tissue colloid osmotic pressures can be either more precisely or more rapidly adjusted by any biological control devices On the other hand both the capillary hydrostatic pressure and the capillary surface area available for the exchange may be subjected to substantial and prompt variations by way of shifts in vascular smooth muscle tone

The capillary hydrostatic pressure is determined by several factors and their mutual relationship is expressed in the formula

$$Pc = \frac{Pa\frac{rv}{ra} + Pv}{1 + \frac{rv}{ra}}$$

(Pappenheimer and Soto Rivera 1948) where Pc Pa and Pv denote the capillary arterial and venous pressures respectively and rv and ra the post capillary and precapillary flow resistances respectively A rise in arterial pressure venous pressure or in the quotient of post to precapillary resistance will increase capillary pressure and hence tend to induce a net filtration of fluid from the blood into the interstitial space Conversely a reduction of the arterial or the venous pressure or of the quotient of post to precapillary resistance will lower the capillary pressure thereby leaving the colloid osmotic forces relatively unopposed promoting an absorption of fluid into the blood stream

By appropriate adjustment of the various factors in the above formula it is possible to induce changes in capillary pressure and thereby in transcapillary fluid exchange However the majority of the cardiovascular control mechanisms seems to be designed to keep arterial and central venous pressures as *constant* as possible Obviously therefore shifts in these pressures cannot be expected to comprise the primary mechanisms by which receptor controlled adjustments of the capillary pressure are induced No doubt any fall in arterial or central venous pressure in connection with for example a blood loss would tend to lower capillary pressure and facilitate a fluid transfer into the cardiovascular system However such a type of fluid gain is then

were found to augment plasma volume although relatively slowly. Studies in humans have given similar results. Thus EBERT, STEAD and GILSON (1941) found a 20 per cent restoration of plasma volume after blood losses comprising 750—1,000 cc and KAUFMANN and MÜLLER (1958) report 50 per cent restoration of the shed blood volume (600—700 cc) in healthy blood donors within 15—20 minutes.

These studies and similar ones show clearly that considerable fluid volumes can be mobilized from the extravascular spaces within a relatively short period of time. They have also shown that a *complete* restoration of the plasma volume requires a substantial period of time. The rate of plasma volume augmentation was thus considerably decelerated after the initial 20—30 minutes following hemorrhage. This fact indicates that the transcapillary fluid transfer must in a sense be considered a selflimiting process. Probably several factors contribute to this. For instance the continuous dilution of the plasma proteins and the eventual concomitant concentration of the tissue colloids can thus be expected to reduce the transcapillary osmotic pressure gradient and hence reduce progressively the reabsorption pressure.

It should be pointed out however that the essential factor responsible for the absorption of fluid into the blood stream after a hemorrhage has generally been considered to be the accompanying reduction of arterial and venous pressures. On the other hand a more moderate hemorrhage is not necessarily followed by a significant fall in arterial and venous pressures (see *e.g.* MEEK and EYSTER 1921, CHIEN 1958) but nevertheless a fluid reabsorption occurs. The experiments by CHIEN (1958) suggest that nervous factors must in some way or other be of importance for the mobilization of extravascular fluid in hemorrhage because the rate of posthemorrhagic hemodilution was significantly reduced after complete sympathectomy despite the fact that hemorrhage caused a more pronounced blood pressure fall in these animals. Exactly how the sympathetic nervous system might have influenced the plasma replacement was not revealed by this study. Recent monographs dealing with the blood volume and its control (for ref. see above) leave this question open though it is assumed that adjustments of vascular tone must somehow be of importance. However with reference to the theoretical discussion outlined above concerning neurogenic adjustments of the pre- to postcapillary resistance ratio mechanisms along these lines seem to offer a reasonable explanation of CHIEN's observation.

Information is more sparse concerning the effects of a sudden increase in blood volume upon the transcapillary net fluid exchange. SMITH *et al.* (1959) found that plasma volume decreased by approximately 20 per cent within 20 minutes after gross blood transfusions. LAWSON and REHM (1943) allowed

The principal factors which according to Starling should be responsible for the net fluid exchange across the capillary membranes have thus been experimentally substantiated and his hypothesis is generally accepted (cf LANDIS and PAPPENHEIMER 1963) On the other hand little is known whether any of these factors can be influenced by suitable control mechanisms so that there would be a true regulation of the fluid partition between the intra and extravascular spaces with the primary aim of maintaining a constant blood volume It seems reasonable that neither tissue pressure nor plasma and tissue colloid osmotic pressures can be either more precisely or more rapidly adjusted by any biological control devices On the other hand both the capillary hydrostatic pressure and the capillary surface area available for the exchange may be subjected to substantial and prompt variations by way of shifts in vascular smooth muscle tone

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a consequence of a failing reflex compensation and has nothing to do with a true reflex control of mean capillary pressure.

On the other hand, reflex resettings of the relationship between the flow resistance proximal to the capillary level and the resistance distal to it — *the pre- to postcapillary resistance ratio* — would provide an effective means for inducing variations in mean capillary pressure within fairly wide limits without necessitating any concomitant changes in either arterial or central venous pressures. As both the pre- and postcapillary vascular sections are supplied with vasoconstrictor fibres, at least in most vascular beds (cf. FOLKOW 1956), reflex changes of the flow resistance in both sections may be readily induced. Whenever the resistances in these two vascular sections are affected to a different degree, a shift in the ratio will occur, resulting in a change in capillary pressure. That such a vascular response pattern can be induced by *direct* stimulation of the regional vasoconstrictor fibres, at least in a skeletal muscle vascular bed, has been shown by MELLANDER (1960). With this type of nerve stimulation, precapillary resistance consistently increased more than postcapillary resistance, which resulted in a lowered mean capillary pressure and a reabsorption of fluid across the capillary membranes. However, little is known whether the same pattern of constrictor fibre discharge and vascular response takes place when the vasoconstrictor fibres are activated by the vasomotor centre and its controlling receptor stations, although some data might suggest that this is the case (FICHSA and WILKINS 1942).

The rate of transcapillary fluid transfer is dependent not only upon the magnitude of the pressure gradients, but also on the functional state of the capillary membrane, both the *permeability (i.e. number and dimensions* of the capillary pores per unit capillary area) and the surface the of size area available for the filtration process (i.e. the number of capillaries open to blood flow). It appears that capillary permeability is not affected by normal vasodilator metabolites (see e.g. LANDIS and PAPPENHEIMER 1963) or by the vasomotor system (CHENG 1949, FOLKOW 1956). It is altered when substances such as histamine (see e.g. SPECTOR 1958) or bradykinin (ELLIOT, HORTON and LEWIS 1960) are released locally, or when there is a gross distension of the capillaries (WASSERMAN, LOEB and MAYERSON 1955, SHIRLEY *et al.* 1957). The size of the effective capillary filtering area can, however, be varied by adjustments of the tone of the precapillary sphincter sections. Their smooth muscles, situated at the entrance to the capillaries, generally induce a complete luminal closure upon activation. This excludes the corresponding capillaries from the active circulation and hence very soon from any significant participation in the transcapillary filtration-reabsorption process (cf. COBBOLD *et al.* 1963). That

the tone of the precapillary sphincters can be affected by for example vasomotor nerve activation seems to be clear from several investigations (e g BUCHERL and SCHWAB 1952 FOLKOW and MELLANDER 1960 ROSELL and UVNAS 1962 RENKIN and ROSELL 1962 COBBOLD et al 1963 FOLKOW et al 1964 b) A mechanism for reflex adjustment of the functional filtering area of the capillary membrane is thus present

It is obvious from the above discussion that neurogenically induced adjustments in the tone of the consecutive functionally different sections in a vascular bed might represent a powerful means by which the net transcapillary fluid exchange can be reflexly affected The capillary hydrostatic pressure may thus be varied within wide limits by appropriate neurogenic shifts in the pre and postcapillary resistances and the direction and rate of the net transcapillary fluid transfer correspondingly influenced Alterations of the activity level or tone of the precapillary sphincters can influence the size of the capillary surface area that at any given moment is available for the filtration — absorption process and will therefore affect the rate of trans capillary fluid exchange

b Earlier studies

There is an abundance of convincing experimental evidence that a redistribution of the extracellular fluid occurs e g after hemorrhage leading to a compensatory augmentation of the plasma volume at the expense of the interstitial fluid volume The degree and rate of the dilution of the red cells and plasma proteins owing to the addition of protein poor interstitial fluid to the circulation have been used as a quantitative and qualitative measure of the transcapillary fluid transfer No comprehensive survey of the vast literature in this field will be given here and for such purposes the reader is referred to recent review articles (GREGERSEN and RAWSON 1959 REEVE 1960 SJOSTRAND 1962) A few more important individual contributions should be mentioned For instance ADOLPH GERBASI and LEPORE (1933) found in dogs that within approximately 20 minutes after a hemorrhage (20—35 cc/kg bodyweight) there was a 35 per cent replacement of the plasma volume LAWSON and REHM (1945) found in anesthetized dogs a fluid replacement comprising 0 15 ml/kg bodyweight/min when they withdrew blood gradually to reach a blood pressure level of 20 mm Hg KAUFMANN et al (1956) found a 30—40 per cent restitution of the shed blood (5—15 ml/kg) within 15—20 minutes in anesthetized splenectomized dogs CHIEN (1958) using unanesthetized dogs found a rapid restoration of plasma volume the rate of which was correlated with the amount of shed blood Even small blood losses (5—10 per cent of total blood volume)

were found to augment plasma volume although relatively slowly. Studies in humans have given similar results. Thus EBERT, STEAD and GIBSON (1941) found a 20 per cent restoration of plasma volume after blood losses comprising 750—1,000 cc and KAUFMANN and MÜLLER (1958) report 50 per cent restoration of the shed blood volume (600—700 cc) in healthy blood donors within 15—20 minutes.

These studies and similar ones show clearly that considerable fluid volumes can be mobilized from the extravascular spaces within a relatively short period of time. They have also shown that a *complete* restoration of the plasma volume requires a substantial period of time. The rate of plasma volume augmentation was thus considerably decelerated after the initial 20—30 minutes following hemorrhage. This fact indicates that the transcapillary fluid transfer must in a sense be considered a selflimiting process. Probably several factors contribute to this. For instance, the continuous dilution of the plasma proteins and the eventual concomitant concentration of the tissue colloids can thus be expected to reduce the transcapillary osmotic pressure gradient and hence reduce progressively the reabsorption pressure.

It should be pointed out, however, that the essential factor responsible for the absorption of fluid into the blood stream after a hemorrhage has generally been considered to be the accompanying reduction of arterial and venous pressures. On the other hand, a more moderate hemorrhage is not necessarily followed by a significant fall in arterial and venous pressures (see *e.g.* MEEK and EYSTER 1921, CHIEN 1958) but nevertheless a fluid reabsorption occurs. The experiments by CHIEN (1958) suggest that nervous factors must in some way or other be of importance for the mobilization of extravascular fluid in hemorrhage, because the rate of posthemorrhagic hemodilution was significantly reduced after complete sympathectomy, despite the fact that hemorrhage caused a more pronounced blood pressure fall in these animals. Exactly how the sympathetic nervous system might have influenced the plasma replacement was not revealed by this study. Recent monographs dealing with the blood volume and its control (for ref. see above) leave this question open, though it is assumed that adjustments of vascular tone must somehow be of importance. However, with reference to the theoretical discussion outlined above concerning neurogenic adjustments of the pre- to postcapillary resistance ratio, mechanisms along these lines seem to offer a reasonable explanation of CHIEN's observation.

Information is more sparse concerning the effects of a sudden increase in blood volume upon the transcapillary net fluid exchange. SMITH *et al.* (1959) found that plasma volume decreased by approximately 20 per cent within 20 minutes after gross blood transfusions. LAWSON and PEHM (1945) allowed

time for plasma dilution after hemorrhage and then replaced the shed blood With this there was a loss of plasma presumably into the tissues For neither study was any explanation of the mechanism involved given

Studies designed to provide more detailed information concerning the exact mechanisms responsible for the adjustment of the transcapillary net fluid exchange and the participation of various cardiovascular reflex mechanisms in this process do not appear to have been undertaken to judge from recent review articles (GREGERSEN and RAWSON 1959 REEVE 1960 and SJOSTRAND 1962) In 1940 GREGERSEN performed a study in which the effect of bilateral carotid occlusion upon the size of blood volume was investigated The arterial blood pressure was markedly elevated in these experiments and if pre and postcapillary resistances had been uniformly increased one should have expected a capillary pressure rise a loss of circulatory fluid into the tissue spaces and consequently a blood volume reduction However no substantial change of blood volume was observed On closer view this suggests that the capillary pressure had not been significantly altered despite the elevated arterial pressure These experiments might in fact be taken as indirect evidence that a shift or more specifically a rise in the pre to postcapillary resistance ratio occurred upon carotid occlusion which — according to the formula above — would tend to counteract the effect of a raised arterial pressure upon the capillary hydrostatic pressure However the data from these experiments are not sufficient to clarify such problems and other mechanisms may also have been involved such as a purely local autoregulatory increase of this ratio (FOLKOW and ÖBERG 1961)

c *Aim of the present study*

In the present study attempts have been made to elucidate whether various cardiovascular reflex patterns could affect the net fluid transfer across the capillary walls in a consistent fashion in which vascular circuits it takes place and to what extent it occurs In the majority of the present experiments the carotid sinus baroreceptor and carotid body chemoreceptor reflex mechanisms were studied Attempts were also made to analyze the effects of activation of the receptors situated in the walls of the different heart chambers These receptors especially those in the left atrial wall have been ascribed a strategic location for sensing variations in blood volume and hence to act as volume receptors (GAUER and HENRY 1963) It was therefore considered to be of interest to analyze whether the transcapillary exchange process with its direct bearing upon the blood volume might be affected by these receptors Lastly the more integrated reflex response pattern induced by sudden re

ductions of blood volume was also studied with special regard to the effects on the transcapillary fluid exchange when all the cardiovascular receptor sites were intact or when some of them were selectively eliminated

It was assumed *a priori* that — if the total plasma volume could be substantially affected by reflex adjustments of the transcapillary net fluid exchange — such adjustments should be of special importance in organs possessing a large tissue mass and hence a considerable volume of extravascular fluid Small organs with a small absolute amount of tissue fluid cannot be expected to play an important role in the overall regulation of fluid distribution between the intra and extravascular compartments Further it is reasonable to assume that more significant transcapillary net fluid movements cannot take place in rigid or rigidly enclosed organs such as the brain or the skeleton Here the tissue pressure can be expected to change markedly upon even a small fluid mobilization or accumulation and will therefore counteract any tendency toward shifts in capillary filtration or absorption For these reasons interest was focused upon skeletal muscle and skin as these tissues comprise about 50 – 60 per cent of the total body weight in cats and in humans (Skelton 1927) and on the intestine since it was thought to be representative of the relatively voluminous abdominal visceral organs

Preliminary reports of these experiments have been presented elsewhere (Öberg 1963 *a* *b*)

CHAPTER II

Methods

a General considerations

In studies concerned with the reflex control of the circulation one must bear in mind that the magnitude of the induced cardiovascular responses is dependent upon the integrity and excitability of all of the components in the complex reflex arcs, i.e. the receptors, their afferent connections and relay stations in the bulbar vasomotor centre (VMC), the tonically active efferent pathways and the various effectors. The functional state of any of these components, and particularly of the vasomotor centre, can be significantly affected as a result of the various experimental procedures necessary for a detailed analysis of cardiovascular reflex mechanisms. The choice of anesthetic and the dosage given must be considered, since e.g. too deep an anaesthesia will markedly suppress the activity and excitability of the VMC. Further, its normal function requires an adequate blood supply and an optimal blood composition with regard to e.g. oxygen and carbon dioxide content. Therefore, blood and fluid losses must be compensated for by sufficient amounts of blood and fluid substitutes, and the pulmonary ventilation of the animal must be such as to ensure a normal gas exchange in the lungs. Whenever artificial respiration is administered care must be taken to match the normal spontaneous frequency and tidal volume as closely as possible to prevent hypo- or hyperventilation and the consequent shifts in blood composition. In addition, the temperature of the animal must be steadily controlled. — In the present study measures were taken to keep all these factors under control as far as was possible so that, as a rule, brisk vasomotor reflexes from the different receptor areas could be elicited.

The vascular responses to a given receptor stimulus are not only dependent upon the integrity of the various nervous links, but are also quantitatively related to the prevailing functional state of the vascular smooth muscle effectors. The general characteristic of autonomic neuro-effectors is that even small shifts in vasomotor fibre activity in the physiological range, which is indeed fairly narrow, induce pronounced vascular reactions (FOLKOW 1952, CELANDER 1954). However, if the prevailing constrictor fibre discharge is very low, reflex inhibition can produce only a small decrease in vascular tone, while a reflex intensification of the discharge rate can produce a very intense

vasoconstriction. Conversely, if the tonic vasoconstrictor fibre activity is high, the ability to induce additional reflex vasoconstriction is limited, whereas a reflex inhibition of this tonic discharge can produce a profound vasodilatation. For such reasons a proper quantitative evaluation of reflexly induced vascular responses necessitates that the initial level of vasoconstrictor fibre activity and vascular smooth muscle tone is at least approximately identified. This is especially important when one wishes to compare the extent of engagement of the different consecutive vascular sections in various cardiovascular reflex adjustments. It has been shown (MELLANDER 1960) that the relation between vasoconstrictor fibre discharge rate and extent of effector response is not identical for the capacitance and resistance vessels. The capacitance vessels exhibit a nearly maximal response at impulse frequencies as low as 4—5/sec while the resistance vessels do not display a maximal constriction until the discharge rate is increased to some 10—15 imp./sec. This means that alterations of the constrictor fibre activity within the low frequency range (0—5 imp./sec) will cover almost the whole scale of obtainable capacitance vessel responses, while only a fraction of the potential resistance response will have been covered. Within the higher discharge range (from 5—10 imp./sec), on the other hand, virtually no additional reflex constriction of the capacitance vessels can be expected, while the reflex resistance response can then be considerably enhanced. These principles must always be borne in mind when attempts are made to evaluate the extent and pattern of reflex shifts in vasoconstrictor fibre activity from the induced effector responses. What might look like a differentiated fibre discharge pattern, as judged from the extent of engagement of the capacitance and the resistance vessels, may in fact be a uniform one, or *vice versa*, depending entirely upon within which part of the physiological discharge range the reflex shifts in fibre activity occur. However, once one has become familiar with these problems, fairly correct evaluations concerning the general extent and the approximate pattern of vasoconstrictor fibre activation or inhibition can be made from the recorded effector responses. Electrophysiological techniques, which theoretically should afford a better approach to such evaluations, seem to be less feasible to use for such purposes, for reasons discussed by LÖFVING (1961 *b*, p. 14—15).

Other complicating factors must also be taken into account if one is to estimate with any degree of exactness the extent of reflex excitation or inhibition of the contractile elements in the different vascular sections. For instance, the reactivity of the vascular smooth muscles can be markedly modified by non-neurogenic influences, *e.g.* by an excessive accumulation of vasodilator metabolites or by circulating vasoactive substances. Such influences are not necessarily equally potent on different vascular sections

(LEWIS and MELLANDER 1962). Furthermore, alterations of effective blood viscosity known to occur when flow rate and vascular dimensions are changed (cf. BAYLISS 1962) can affect flow resistance and may lead to erroneous estimations of the resistance vessel responses. More abnormal viscosity changes caused e.g. by erythrocyte sludging within venules and small veins may increase the *postcapillary* flow resistance enough to raise capillary pressure and disturb the fluid equilibrium across the capillary membranes even though *total* flow resistance is very little altered.

Finally, changes in the transmural pressure *per se* will affect the dimensions of the blood vessels and hence their flow resistance and blood content. This will be especially marked in the venous section where the vascular distensibility is high and where fairly pronounced shifts of blood content can occur with only small changes in the distending pressure. Whenever a vasoconstriction occurs proximal to the veins, there will be a decrease in venous transmural pressure. This will cause a passive elastic recoil of the veins and an expulsion of blood from this region. Consequently, the blood expulsion from the veins observed when the vasomotor fibres to an organ are activated is due both to active shortening of the smooth muscle cells in the capacitance vessels and to passive elastic recoil. The relative magnitude of the passive emptying in this situation seems to depend upon the prevailing distending pressure in the capacitance vessels. If the distending pressure is initially low, a further pressure reduction may produce a collapse of the veins, leading to a very pronounced passive emptying of blood. A superimposed neurogenic constriction of the veins at that time would not be able to squeeze out any significant amount of blood from the nearly empty capacitance section and the erroneous conclusion might be drawn that the nervous control of this vascular section is negligible. Conversely, if the venous transmural pressure is initially high, the same transmural pressure reduction will not cause any collapse of the veins, and the passive elastic recoil caused by a vasoconstriction proximal to the veins will then expel only a small amount of blood. In this situation, however, an active constriction of the venous smooth muscles will be able to produce a very marked "active" blood expulsion and will thus clearly illustrate the true influence of the neurogenic control of the venous compartment. A systematic analysis of these problems concerning venous control is beyond the scope of the present communication, but will be dealt with in another publication (ÖBERG 1964). In the present study venous outflow pressure was kept relatively high, which will tend to minimize the changes in venous transmural pressure upon vasoconstrictor fibre activation and a collapse of the veins can be avoided. By analogy with the analysis performed by MELLANDER (1960), the reflexly induced changes in regional blood content in this study

can be assumed to be due mainly to active venous constriction and they therefore closely reflect the extent of the direct neurogenic influence upon the venous compartment.

The considerations briefly outlined above emphasize the multiplicity of factors which must be taken into consideration in analyzing the reflex control of the cardiovascular system. It is then not surprising to find that sometimes fairly pronounced quantitative differences in the extent of the vascular responses were obtained in different animals or even in the same animal during the course of the experiment. For such reasons this study was not primarily designed to present a full quantitative analysis of the vascular adjustments induced by the various cardiovascular reflex mechanisms which were studied. However, the quantitative data given may provide approximate information on the magnitude of the vascular adjustments and the rate of the transcapillary net fluid transfer that can be induced reflexly.

In the present study qualitative and quantitative information concerning the net transcapillary fluid exchange in various organs was assessed by a volumetric method previously described and critically analyzed by MELLANDER (1960). Alterations in the volume of an organ can be due to changes in its blood content and/or in its extravascular fluid content. Changes in the amount of blood will reflect shifts in vascular tone, primarily in the capacitance vessels, while shifts in the quantity of extravascular fluid will indicate changes in the net fluid exchange across the capillaries, provided that no lymph drainage from the tissues occurs. In most instances alterations in blood volume and extravascular fluid volume can be differentiated and studied separately due to their distinct time characteristics. Blood expulsion or accumulation as a result of venous constriction or dilatation, respectively, *will thus be* revealed as a rapid phasic change of tissue volume, synchronous with other manifestations of adjustments of vascular tone such as changes of blood flow resistance. On the other hand, the transcapillary fluid transfer is a relatively slow process and will then present itself as a slow and fairly steady volume change continuing long after the phasic shifts in tone of the resistance and capacitance vessels have been completed. The correctness of such an interpretation is supported by the original analysis of MELLANDER (1960) and by later experiments in this laboratory in which the plethysmographic method was combined with an isotope technique for measuring blood content separately by means of labelled erythrocytes (ÅBLAD and MELLANDER 1963, KJELLMER, personal communication). — In the present study, therefore, slower volume changes which occurred during steady state blood flow conditions and then continued relatively undisturbed both as to magnitude and

direction for a longer time period have been considered to reflect shifts in the net transfer of fluid across the capillary membranes

Appropriate adjustments of the different series coupled sections in a vascular bed appear as discussed above to constitute the essential mechanism by which the transcapillary net fluid exchange may be affected during various cardiovascular reflex responses Therefore attempts were made to study separately and in more detail the reflexly induced reactions of the pre and post capillary resistance vessels the precapillary sphincters and the capacitance vessels in different tissues This was accomplished by combining the recording of tissue volume with continous recordings of regional blood flow and of arterial inflow and venous outflow pressures Changes in regional flow resistance which can then be calculated will reflect the reactions of the resistance vessels and then mainly of the precapillary ones since they constitute by far the greatest part of the total resistance (PAPPENHEIMER and SOTO RIVERA 1948) From the rapid phasic volume changes the reactions of the capacitance vessels can be followed Further by keeping the arterial inflow and venous outflow pressures constant reflex changes of the pre to post capillary resistance ratio will be revealed by a shift in the net transcapillary fluid exchange and the consequent slow but steady tissue volume change the rate of which will be approximately related to the extent of the reflex resetting of this ratio Finally repeated determinations of the capillary filtration coefficient (CFC) will provide information whether and to what extent the capillary surface area available for the fluid exchange has been reflexly changed by shifts in the activity of the precapillary sphincters By measuring CFC and the rate of transcapillary fluid exchange it is possible to estimate the extent to which mean capillary pressure has been altered during the various experimental procedures The extent of reflex shifts in the pre to post capillary resistance ratio can then be estimated more correctly

b Material anesthesia and general operative procedures

84 cats with body weights varying between 1 5 to 4 0 kg have been used in this study After preliminary administration of ether prolonged anestesia was usually induced with chloralose 25 to 50 mg/kg body weight In some animals chloralose urethane (30—50 mg/kg and 100 mg/kg respectively) or nembutal 25—30 mg/kg was used However since the general excitability level of the cardiovascular reflexes was usually quite good when chloralose alone was used this anaesthetic was considered to be the most suitable one for the present experiments — A tracheal cannula was inserted Body temperature was kept at 37 to 38 C by means of a thermostatically regulated heating pad

The carotid arteries and the vagosympathetic trunks in the neck were cautiously dissected free for further preparations. After the completion of the special operative procedures for the recording of the various circulatory parameters etc. (see below) the animal was as a rule given artificial respiration by means of a Starling "Ideal" pump, the frequency and tidal volume of which was adjusted as closely as possible to the normal spontaneous respiration. — When an adequate ventilation was ensured the animals were generally *curarized* with gallamine triethiodide (Flaxedil ©, May and Baker) to avoid disturbances of the tissue volume recordings from undue muscular movements. Flaxedil was given in amounts of 2—4 mg/kg body weight as an initial dose and in supplementary doses of 0.5—1 mg/kg when needed during the experiment.

When the intestinal preparation was used and when the heart receptors were stimulated by injections of veratrum alkaloids (see below) *atropine* was usually given intravenously 0.3—0.4 mg/kg. *Heparin* was used as anticoagulant, 5 mg/kg was given as an initial dose, supplemented if necessary during the experiment. — Blood losses were replaced by a *dextran Tyrode* solution. A dextran preparation with a low average molecular weight (40 000) and a narrow molecular weight distribution (Rheomacrodex ©, Pharmacia) was used in this study. This dextran preparation does not affect the suspension stability of the blood significantly and is therefore considered to be a suitable plasma expander in cats (ELIASSON and SAMELIUS BROBERG 1963). — In some experiments reflex changes in the *adrenomedullary output of catechol amines* was prevented by ligation of the right and denervation of the left suprarenal glands. The steady secretion of cortical hormones from the left gland will not be significantly disturbed by these procedures.

c. Special operative procedures

In this study transcapillary fluid exchange was studied in skeletal muscle, skin and intestine. The hindquarters of the animal after exclusion of the paws and the tail, or the calf were considered representative *skeletal muscle preparations* although with the techniques used in this study a certain amount of skin and skeleton was unavoidably included in the preparation. The paw of the cat, after exclusion of the pads, was chosen when the reactions of a *skin vascular bed* were to be studied. Also in this preparation skeleton and a small amount of skeletal muscle and tendons will be included. The reflex adjustments of the *intestinal vascular bed* were studied in a loop of the small intestine, usually about 50 grams of tissue.

When the *hindquarters* of the animal were the subject of study the preparation followed the technique described earlier in detail by MELLANDER (1960).

The abdomen was opened by a midline incision and the intestine as well as the spleen and the great omentum were extirpated to allow free access to the aorta and the inferior caval vein. These vessels were carefully dissected free from the surrounding tissue and all side branches between their bifurcations and the renal vessels were ligated and divided. To ensure that all venous blood from the hindquarter preparation drained *via* the inferior caval vein, efforts were made to occlude all other draining channels. Thus the blood flow along the abdominal muscles, the muscle bundles on the back and around the vertebral column was obstructed by means of mass ligatures. Further, a hole was drilled in the spinal column at the height of the fifth lumbal vertebra and the spinal canal was plugged with cotton soaked in vaseline so as to obstruct the intraspinal veins. This latter procedure will also interrupt practically all of the somatomotor fibres running to the region but will not disturb the sympathetic preganglionic fibres as these leave the spinal cord at a more rostral level. During the preparation great care was taken to protect the sympathetic trunks from cooling, drying and from mechanical damage. With this procedure the hindquarters of the animal were separated from the rest of the animal except for the aorta, the caval vein and the sympathetic nerve supply. The lymph drainage from the hindquarters was thus also obstructed. The ureters were, however, generally left intact to permit free drainage of urine into the bladder, which was emptied through a wide bore cannula inserted through the bladder wall. The anus, the urethra and in female cats the vagina also were closed with sutures. The tail and the paws were excluded from the circulation by means of firm ligatures. The hindquarters were then placed in a plethysmograph. To ensure complete tightness of the plethysmograph, a circular skin flap was dissected free from underlying tissues at the division line between the upper part of the animal and the hindquarters so as to form a skin collar around the opening of the plethysmograph. A flange covered with a thin layer of vaseline was placed over the skin collar and fixed firmly to the opening of the plethysmograph with screws. The plethysmograph was filled with water, the temperature of which was kept at 37 to 38° C by means of an external heat lamp and controlled with an electric thermometer (Electric Universal thermometer, type TE 3, Electrolaboratoriet, Copenhagen).

In ten cats the proportions of the different kinds of tissue of the hindquarters were determined by weighing them separately after the experiment. Skeletal muscle comprised on the average 52 per cent (47—56 per cent) of the preparation, skin on the average 20.5 per cent (16—25 per cent) and skeleton 28.3 per cent (25—31 per cent).

The preparation of the *calf* followed in principle the same procedure. The skin around the upper part of the thigh was incised and cautiously dissected

free from the underlying musculature down to the knee joint. At this level all muscle bundles were severed with careful hemostasis. A hole was drilled in the femur and the medullary cavity was plugged. The calf was thus separated from the rest of the animal except for the cognate artery and vein and the sciatic nerve which contains the great majority of the vasomotor nerves to the calf muscles. Care was taken to protect these structures from cooling, drying or mechanical damage. The paw was excluded from the preparation by disarticulation at the ankle joint. The calf was placed in a specially designed plethysmograph using the dissected skin collar as a watertight seal. The water temperature in the plethysmograph was held at 37 to 38° C. The exposed muscles on the thigh were covered with gauze soaked in saline and over this was placed a plastic sheet to prevent cooling and evaporation. The proportion of the different types of tissues of the calf was measured in 5 cats. Skeletal muscle comprised on the average 55 per cent (51—57 per cent) of the preparation, skin 14 per cent (13—16 per cent) and skeleton 31 per cent (27—35 per cent).

The reactions of the *cutaneous* vessels were studied in the paw which was prepared in much the same way as the above procedure. Thus a skin collar was dissected free from the calf down to the ankle joint. At this level a hole was drilled in the tibia and the medullary cavity was obstructed with cotton plug. All other tissues except for the nerves, the artery and the great saphenous vein were severed. In most experiments the pads were excluded from the preparation by firm ligatures around their bases since their blood flow to a great extent courses through specialized arterio-venous anastomoses which subserve the temperature regulation. The paw was placed in a plethysmograph and the skin collar was used as a watertight seal. The temperature of the water in the plethysmograph was kept constant at 37—38° C. The proportion of the different tissues in the paw was determined in 5 experiments. Skin comprised 33 per cent (28—36 per cent), tendons and muscle 19 per cent (9—11 per cent) and skeleton 48 per cent (53—63 per cent).

The *intestinal preparation* was carried out according to the technique described by FOLKOW, LUNDGREN and WALLENTIN (1963). The abdomen was opened in the midline and a loop of suitable length of the small intestine (usually weighing 40 to 60 g) was isolated from the rest of the gastrointestinal tract by means of ligatures. The rest of the gut, the spleen and the great omentum were extirpated. The mesenteric root carrying the vessels and nerves was freed from adjacent tissues so that a fairly long flexible mesenteric pedicle was made available. Great care was taken not to damage the vessels or the nerve fibres during this procedure. The intestinal loop was placed in a triangular perspex plethysmograph with the mesenteric pedicle carefully

positioned in its well fitting opening. To ensure a completely watertight closure of the plethysmograph the opening was sealed with a waterrepellent grease (Plastibase ® Squibb). It was unavoidable that a certain amount of external pressure was exerted upon the structures in the pedicle when the plethysmograph was closed. To prevent a collapse of the draining vein on closing the plethysmograph the cannula used to divert the venous outflow into the blood flow recorder (see below) was advanced into the mesenteric vein so that its opening lay well inside the plethysmograph. The lymph vessels from the intestine can, however, be expected to be essentially occluded by this external pressure. In experiments where the lymphatics in the mesenteric pedicle could be easily recognized they were ligated. The plethysmograph was filled with Tyrode solution kept at 38° C by means of an external heat source. — The intestinal preparation also included the mesenteric lymph glands, the weight of which constituted on an average 15 per cent of the total weight of the preparation.

d. Recordings of cardiovascular parameters

The arterial blood pressure was recorded with a mercury manometer connected to the inferior mesenteric or left renal artery for the hindquarter and intestinal preparations and to one femoral artery for the paw and calf preparations. When the vascular responses of both paw and calf were studied simultaneously in the two hind limbs the arterial pressure was as a rule measured in the inferior mesenteric artery made accessible through a small abdominal midline incision.

The *regional blood flow* of the organs studied was measured by cannulating the respective draining veins, thus diverting the entire venous effluent through a flow recording device. Wide bore catheters were used for the flow recordings to minimize the resistance of the recording device. The flow recorder consisted in most experiments of a perspex drop chamber filled with silicone oil, the drops being registered by a photocell device operating an ordinate writer (CLEMENTZ and RYBERG 1949, LINDGREN 1958). The heights of the ordinates of the writer are inversely proportional to the volume of blood flow. The blood flow in ml/min could be obtained from calibration curves determined for each set of drop chamber-ordinate writer unit. When the hindquarter preparation was used the blood flow was often too big to be measured accurately by a drop chamber device. In these experiments a modified Gaddum recorder (GADDUM 1929) connected to a volume piston recorder was used. The length of the collecting phase of the Gaddum recorder could be varied so as to permit accurate recordings of a wide range of blood

flows The height of the piston recorder excursion is proportional to the blood flow The accuracy of the flow recorders were intermittently checked during the experiments by collecting the outflowing blood for 10 sec in a graduated cylinder

After passing through the flow recorders the blood was returned to the animal *via* a funnel connected to the jugular or left renal vein or when the hindquarter preparation was used to the central end of the inferior caval vein The funnel was equipped with a simple inflow control system by which the rate of inflow of blood to the animal always matched the rate of outflow *via* the blood flow recorders In this way the amount of blood in the funnel and hence in the extracorporeal loops could be maintained almost constant despite large variations in blood flow

The venous outflow pressure in the organs studied was determined by the level at which the Gaddum recorder or the free ends of the tubings draining the drop chambers were placed above the studied organ By adjustments of this height the venous pressure could be set at any desired level during the experiment or kept constant at a known value At the beginning of the experiment the venous outflow pressure was so adjusted that the tissue volume remained constant (*isovolumetric state*) indicating that no net transcapillary fluid transfer occurred Therefore according to the Starling hypothesis there existed a transcapillary pressure equilibrium The venous pressure was then kept constant at this level during each experimental procedure except when CFC was determined (see below)

In most experiments it was also desirable to keep the arterial inflow pressure constant This will eliminate such changes in blood flow and in capillary pressure which are secondary to a changed pressure gradient across the vascular bed The neurogenically induced shifts in tone in the various vascular sections will then be more clearly revealed as will the consequent shifts in transcapillary fluid transfer In most experiments the inflow pressure was kept constant simply by adjustments of a screw clamp placed around the aorta proximal to the arterial branch which supplied the organ and also proximal to the site of the blood pressure recording In other experiments a blood pressure compensator in essence similar to that described by WINDER (1938) was used It consisted of a closed blood reservoir connected to a suitable artery and to a tank with compressed air By adjustments of an outlet valve a constant pressure of any desired value could be maintained in the reservoir The pressure in the reservoir was a rule adjusted to a value corresponding to the resting arterial blood pressu[illegible] In [illegible] r experiments a cross circulation technique was [illegible] part under study was perfused at a con[illegible] imal

From the recorded arterial and venous pressures and blood flow the *regional flow resistance* could be calculated. It was expressed in peripheral resistance units (PRU) denoting pressure head in mm Hg divided by blood flow in ml/min/100 g tissue.

Changes in tissue volume were recorded by connecting the plethysmograph to a carefully balanced and trimmed volume piston recorder. The sensitivity of the volume recording system could be chosen by using various sizes of piston recorders. When intestinal, calf and paw preparations were used volume shifts of 0.05—0.1 ml corresponding to 1—5 per mille change of tissue volume could be recorded satisfactorily. When the hindquarter preparation was studied a piston recorder giving clearcut and reproducible excursions for a volume change of 0.5 ml corresponding to roughly 1 per mille change of tissue volume was chosen. — The changes in tissue volume are expressed in ml/100 g tissue, skeletal weight excluded.

The *capillary filtration coefficient* (CFC) is defined as the amount of fluid in ml filtered across the capillary membranes per minute in 100 g tissue for each mm Hg pressure difference across the capillary membrane. CFC was determined by elevating the venous outflow pressure a known amount, usually 5—20 cm of water, which allowed a measurement of the rate of net filtration caused by the consequent rise in capillary pressure (for detail see COBBOLD *et al.* 1963). Since the magnitude of the capillary pressure increase ensuing upon a given rise of the venous outflow pressure is determined by the pre- to postcapillary resistance ratio this ratio must in principle be known. However, as this ratio cannot be determined exactly in experiments of this type, it has in this study been assumed to be 4:1 throughout, which is the value normally found in the resting steady state (PAPPENHEIMER and SOTO-RIVERA 1948, COBBOLD *et al.* 1963). This value for the pre- to postcapillary resistance ratio is, however, no doubt changed in many situations. For example, it is increased when there is a raised vasoconstrictor fibre activity (MELLANDER 1960). On the other hand, as has been pointed out by CELANDER and MARILD (1962) and by COBBOLD *et al.* (1963), the estimated CFC when using the figure 4/1 for the pre- to postcapillary resistance ratio will not deviate more than approximately ±12 per cent from the true value, even if the ratio should be as low as 2:1 or as high as 9/1. This must be looked upon as a small error in the present study, in which CFC changes were often quite drastic. CFC has been, according to definition, expressed in ml filtered fluid/min mm Hg pressure difference across the capillary membrane/100 g tissue, skeleton weight excluded.

The various parameters studied were recorded on smoked kymograph paper. In some experiments, however, the arterial blood pressure was recorded on a

direct writing oscilloscope (Grass Polygraph model 5) by means of a Statham pressure transducer (P23 AC) to permit an accurate estimation of the pulse rate. In some experiments the central venous pressure was similarly monitored from an indwelling catheter in the jugular vein and a Statham transducer (P23 BC).

e. Mode of receptor stimulation

In many experiments variations of the carotid sinus baroreceptor activity were induced simply by a bilateral occlusion and gentle tugging of the common carotid arteries. However, in some experiments a Moissejeff preparation (MOISSEJEFF 1926) connected to a pressure reservoir which contained oxygenated Tyrode solution, was utilized. In still other animals one or both carotid sinus regions were perfused with arterial blood *via* an exteriorized loop system through a Sigmamotor pump (Model T M 11). For this blood was taken from the proximal end of one carotid artery and passed through the pump to the cephalic end of one or both carotid arteries. The external carotid arteries were cannulated and connected *via* a tubing to the external jugular vein. By adjustments of a screw clamp on this tubing the outflow resistance from the carotid sinus regions could be varied and the intrasinusal pressure consequently changed over a wide range. The intrasinusal pressure was recorded from a T tube in the carotid artery jugular vein shunt proximally to the screw clamp. To prevent an excess leakage of blood from the carotid sinus region into the animal when the intrasinusal pressure was elevated, the main additional arterial branches from the perfused sinus regions were ligated. A small leakage was present in many experiments as often minor arterial branches had to be left open, but this was not considered to interfere significantly with the operation of the artificially perfused receptor areas. Since the Sigmamotor pump delivers a pulsatile flow, a more physiological type of baroreceptor stimulation could be accompli[illegible] this than with a M[illegible] preparation. — In [illegible] ts an [illegible] amber consisting of an airfilled container [illegible] to th[illegible] from the pump to the perfused sinus [illegible] [illegible] be damped as desired.

The chemoreceptor [illegible]
mulated by utiliz[illegible]
that either arterial [illegible]
and rate through th[illegible]
by connecting the [illegible]
artery but also to a [illegible]

funnel through which the blood from the blood flow recorders was returned to the animal In some experiments the chemoreceptors were stimulated by injecting sodium cyanide (0 05—0 1 mg) into the perfusing system

The receptors located in the heart and adjacent structures were activated in different ways In some experiments a mechanical distension of the left atrium was accomplished without causing obstruction of the blood flow by expanding loops of stiff nylon thread which were introduced into the atrium through a polyethylene tubing tied into the auricle The auricle was approached through a small thoracotomy After the tube had been positioned in the atrium the pneumothorax was reduced by gentle pleural suction and the chest closed — In other experiments the heart receptors were stimulated by injecting protoveratrine 1—3 micrograms (puroverin ® \ Sandoz) into the right atrium through an indwelling catheter inserted through the external jugular vein Injections of the same amount of the drug into the root of the aorta through a catheter introduced via one of the subclavian arteries was performed as a control in some experiments to test whether the arterial receptors could be activated by these small amounts of the drug This was obviously not the case since the very small reflex responses sometimes observed had such a long latency that this would correspond to the time needed for the drug to return to the heart receptors via the systemic circulation

When reflexes from the various individual receptors were studied attempts were made to eliminate counteracting buffering effects of other receptors Thus when the carotid sinus and carotid body receptor reflexes were studied both vagal nerves were generally cut in the neck Similarly when the heart receptor reflexes were studied the sinus and the aortic depressor nerves were usually cut When the carotid body chemoreceptor reflexes were studied with a perfused carotid sinus preparation the intrasinusal pressure was kept as constant as possible to prevent concomitant shifts in baroreceptor activity

CHAPTER III

Vascular adjustments and net transcapillary fluid exchange in various cardiovascular reflexes

The experiments presented in this chapter were designed to provide information whether and if so to what extent the various individual cardiovascular reflex mechanisms were capable of affecting the net fluid exchange across the capillary membranes in different tissues. The result of such analyses will then furnish a background for a proper evaluation of the effects of the more integrated cardiovascular reflex response patterns induced by *e.g.* hemorrhage (Chapter IV).

A. Carotid sinus baroreceptor reflexes

a. General considerations

The arterial baroreceptor reflexes are probably the most powerful means by which appropriate adjustments of the cardiovascular system can be induced whenever the blood pressure tends to fall as a result of hemorrhage, blood pooling etc. The tonic restraint normally exerted by the baroreceptors upon the vasomotor centre will then lessen, leading to an augmentation of the tonic sympathetic discharge to the various peripheral vascular circuits and to the heart, combined with an inhibition of the vagal influence on the heart. Prompt compensatory changes of peripheral vascular tone and of heart efficiency can thus be effected *via* this mechanism.

The main characteristics of the peripheral vascular reactions in baroreceptor reflexes are well known (see *e.g.* HEYMANS and NEIL 1958). An increased flow resistance, reflecting a constriction of the resistance vessels, occurs in most vascular circuits when the baroreceptor restraint on the VMC is reduced or eliminated (HEYMANS and NEIL 1958, LOFVING 1961 *a, b*, MCCUFF and AVIADO 1961, POLOSA and ROSSI 1961). However, the flow resistance does not seem to be affected to the same extent in the various parallel coupled vascular beds. The cerebral and coronary resistance vessels thus seem to be essentially unaffected in baroreceptor reflex adjustments, due at least in part to their sparse supply of vasoconstrictor fibres (cf. FOLKOW 1956, 1960). There is further evidence that *e.g.* limb flow is in general more affected than renal blood flow in baroreceptor reflexes (HARTMAN, ORSKOV and REIN 1937,

OPITZ and SMYTH 1937 McGIFF and AVIADO 1961) LÖFVING (1961 *a*, *b*) found by recording simultaneously blood flow changes in different systemic vascular circuits that shifts in baroreceptor activity affected the resistance vessels in skeletal muscle to a far greater extent than *e g* those in the intestine or the skin while the renal vessels were little or even insignificantly influenced

There is also convincing experimental evidence that a constriction of the capacitance vessels occurs when the baroreceptor activity is lowered (see *e g* HEYMANS and NEIL 1958 ROSS FRAHM and BRAUNWALD 1961 *a*) although a more detailed quantitative analysis concerning the extent of the capacitance responses in the different vascular circuits has not been performed

In contrast to the available information concerning the reflex adjustments of the resistance and capacitance vessels there is no direct experimental evidence that the fluid exchange across the capillary walls can be influenced in baroreceptor reflexes although as mentioned earlier indirect data have been provided by GREGERSEN (1940) Since a number of the cardiovascular adjustments consequent upon blood loss seems to be related to a reduced baroreceptor activity it would be of interest to know whether a mobilization of fluid into the circulation from the tissue spaces might constitute one component of the baroreceptor reflex response pattern If so a potent mechanism for a reasonably rapid restoration of the plasma volume in hypovolemic states would be available

b Results

Skeletal muscle Baroreceptor adjustments in a skeletal muscle vascular bed (hindquarters or calf muscle) were studied in 20 cats Fig 1 illustrates an experiment in which the reflex changes in flow resistance blood content and net capillary fluid exchange were followed in a hindquarter preparation when the two carotid sinus regions were perfused at various pressure levels A sequence of periods with alternating high and low intrasinusal pressure levels was studied The mean intrasinusal pressure was kept relatively low initially (85—90 mm Hg) to ensure that a moderate degree of tonic vasoconstrictor fibre activity was present to make possible both reflex decreases and increases of this prevailing activity Prior to the sequence shown in the figure the arterial inflow pressure was reduced by means of a screwclamp around the aorta to the lowest level expected to be reached when the baroreceptors were subsequently stimulated This was done so that the inflow pressure to the hindquarters could be kept essentially constant during the experiment The venous outflow pressure was adjusted initially so that there existed an isovolumetric state implying a transcapillary fluid equilibrium according to the Starling hypothesis

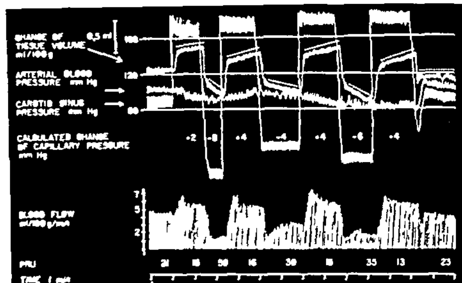


Fig 1 Cat 4 5 kg Chloralose Effects of pressure variations in bilaterally perfused carotid sinus regions on blood flow and tissue volume of the hindquarters The arterial inflow pressure was kept constant during the illustrated procedures by means of an adjustable screw clamp placed around the abdominal aorta The clamp was completely released at the end of the illustrated experimental procedures The venous outflow pressure was initially adjusted so that there was an isovolumetric state — Changes in blood flow and flow resistance (PRU) indicate the reflex affects on the resistance vessels — The initial rapid change of tissue volume with altered baroreceptor activity reflects the reactions of the capacitance vessels while the following slower volume change occurring during steady state blood flow conditions indicates the rate and direction of net transcapillary fluid transfer (dotted lines) — Stimulation of the baroreceptors by elevation of the intra sinusal pressure can be seen to induce a dilatation of resistance and capacitance vessels and a net filtration of fluid from the blood into the tissue spaces The opposite reactions occur when the intrasinusal pressure and hence baroreceptor activity is lowered The reflexly induced shifts in capillary pressure are calculated and given in approximative figures — The animal was curarized and kept under artificial ventilation and the vagal nerves were cut in the neck

The blood flow recording in Fig 1 demonstrates that a stimulation of the carotid sinus baroreceptors by elevating the intrasinusal pressure regularly induced a dilatation of the resistance vessels in the hindquarters revealed as an increased blood flow The flow resistance was thus reduced to approximately 70 per cent of control during the various periods of baroreceptor excitation When the baroreceptor activity was lowered by a reduction of the intrasinusal pressure a constriction of the resistance vessels occurred increasing the flow resistance 1 5 to 2 times control

The registration of tissue volume demonstrates that a sudden elevation of the intrasinusal pressure induced a marked and prompt augmentation of tissue volume After this initial rapid volume increase occurring at the time

of the rapid change in blood flow there followed a phase of slow but fairly steady volume gain which continued as long as the intrasinusal pressure was kept elevated and took place under conditions of constant pressure and blood flow (dotted line) This slow volume increase can be ascribed to a net filtration of fluid from the intravascular into the extravascular compartment according to the interpretation given by MELLANDER (1960) in his experimental analysis of the different phases of the volume shifts (see also Chapter II) The initial rapid volume gain indicates an accumulation of blood in the hindquarters consequent upon a reflex dilatation of the capacitance vessels

One can therefore conclude that an increase of the carotid baroreceptor activity induced a reflex dilatation of the capacitance vessels of the hind quarters and a net filtration of fluid from the intravascular into the extra vascular compartment

Conversely a lowering of the intrasinusal pressure and the consequent in hibition of baroreceptor activity can be seen to induce a constriction of the capacitance vessels and a net absorption of tissue fluid into the blood stream

A quantitative evaluation of the recorded volume changes reveals that approximately 0 6 ml blood per 100 g tissue was mobilized from and again accumulated in the hindquarter capacitance vessels when the mean intra sinusal pressure (and hence the baroreceptor activity) was varied between 10 and 180 mm Hg Since the regional blood volume in the hindquarters amounts to approximately 2 5—3 ml/100 g tissue (MELLANDER 1960) it can be cal culated that about 20 per cent of the regional blood volume constituted a functional blood depot which could be mobilized to enhance venous return to the heart when the vasoconstrictor fibres were reflexly activated by a diminished baroreceptor activity

The amount of fluid transferred from the blood across the capillary mem branes into the extravascular compartment of the hindquarters varied between approximately 0 04 and 0 08 ml/min/100 g tissue during the different periods of elevated intrasinusal pressure The rate was dependent upon the extent of the intrasinusal pressure change When the intrasinusal pressure was lowered between 0 08 and 0 16 ml/min of fluid was absorbed from 100 g tissue into the blood This experiment thus clearly shows that the direction and rate of the net transcapillary fluid exchange in the skeletal muscles can be markedly influenced by carotid sinus baroreceptor reflexes and that a sub stantial amount of fluid can be transferred across the capillary membranes during fairly short periods of time

As discussed earlier the net movement of fluid across the capillary mem branes during baroreceptor reflex adjustments must be ascribed to changes in mean capillary hydrostatic pressure Since in this experiment both the

venous outflow pressure and the arterial inflow pressure were kept essentially constant the capillary pressure shifts must be due to changes in the pre to postcapillary resistance ratio This implies that the ratio was lowered i e the precapillary resistance was decreased to a greater extent both absolutely and relatively than the postcapillary resistance with augmentation of baro receptor activity Conversely a diminution of the baroreceptor activity evidently caused a relatively more marked increase of the precapillary re sistance since mean capillary pressure then always decreased as judged by the net absorption of fluid The approximate shifts in mean capillary hydro static pressure from the isovolumetric value have been calculated (see below) and the values are given in the figure

Fig 2 shows recordings from an experiment performed in a similar way to that described in Fig 1 with the addition that during the course of the experiment the capillary filtration coefficient (CFC) was repeatedly deter mined (signals) This was done in order to provide information as to whether the size of the capillary surface area available for the fluid exchange process, was altered significantly in baroreceptor reflexes Here the arterial blood press ure was kept constant by means of the pressure compensator CFC was deter mined by elevating the venous outflow pressure by 5 cm H_2O (signals), which induced a rapid initial volume gain due to a passive venous distension fol lowed by a slower but steady increase of tissue volume reflecting the rate of net filtration produced by the increase in mean capillary hydrostatic press ure From the rate of this net filtration as related to the capillary pressure increase CFC can be determined (for details see Chapter II) During control conditions (A) CFC was found to be 0 016 ml/mm Hg/min/100 g tissue a value in agreement with previous observations in skeletal muscle (PAPPENHEIMER and SOTO RIVERA 1948 MELLANDER 1960 COBBOLD *et al* 1963) When the carotid sinus pressure was lowered (B) vascular adjustments similar to those described earlier were induced Thus a constriction of the resistance and the capacitance vessels occurred as well as a continuous absorption of tissue fluid into the blood stream When a steady state blood flow and a well defined slope of the volume curve were established CFC was again determined (B) It was now 0 023 or approximately 45 per cent higher than the initial control value In other words in the steady state condition a reflex blood flow *decrease* was associated with an *increase* of CFC in the skeletal muscles It should further be noted that the magnitude of the passive venous distension to the same outflow pressure increase was now (B) somewhat less than in A This implies a reduced distensibility of the capacitance vessels which is another expression of the augmented venous tone occurring as a result of vasoconstrictor fibre activation When the intrasinusal pressure was elevated

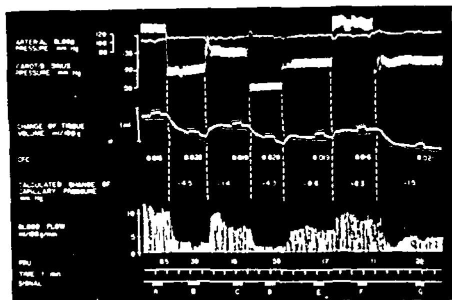


Fig. 2. Cat 3.8 kg. Chloralose. Effects of pressure variations in bilaterally perfused carotid sinus regions on resistance and capacitance vessels, capillary filtration coefficient (CFC) and net capillary fluid transfer in the hindquarters. — CFC is determined from the rate of net filtration caused by an elevation of the venous outflow pressure by 5 cm H_2O (signals). Note that CFC increases with reflex augmentation of the vasoconstrictor fibre activity and *vice versa*. — The animal was curarized and kept under artificial respiration. The vagal nerves were cut in the neck. Arterial blood pressure was kept essentially constant throughout the experiment by a pressure compensator.

(C) a reflex dilatation of resistance and capacitance vessels occured, the rate of fluid absorption was slowed down and CFC was reduced towards the control value. Thus throughout this experiment it was found that CFC regularly changed in a direction *opposite* to flow resistance. This was a constant finding in the five experiments devoted to systematic determinations of CFC in skeletal muscles during baroreceptor reflex adjustments.

As discussed earlier, it is reasonable to believe that the calculated changes in CFC are related to changes in the tone of the precapillary sphincters. A relaxation of this vascular section evidently occurred whenever vasoconstrictor fibre activation reduced the blood flow in skeletal muscles and *vice versa*. This might seem surprising since earlier studies have indicated that the vasomotor fibres in fact exert a constrictor influence on the precapillary sphincters (Folkow and Mellander 1960, Renkin and Rosell 1962). However, the vasodilator metabolites accumulated as a consequence of the neurogenically induced reduction of the blood flow seem to affect the precapillary sphincter vessels especially strongly and overcome the neurogenic influence so as to

produce a net *increase* of the perfused capillary surface area (COBBOLD *et al* 1963) The present findings are in agreement with those of COBBOLD *et al* and imply that whenever a reflex augmentation of vasoconstrictor fibre activity increased the pre- to postcapillary resistance ratio and thereby produced an absorption of fluid from the extravascular space a somewhat increased capillary surface area was available for this fluid exchange

In general CFC was found to increase by *roughly* 10—20 per cent when the hindquarter blood flow was reflexly reduced to half of control by 20—50 per cent when blood flow was reduced to half to one fourth of control and by 40—80 per cent when the blood flow was reduced even more

When CFC is known it is possible to calculate approximately to what extent mean capillary pressure must have departed from the 'isovolumetric value' to produce a given net transfer of fluid during baroreceptor reflex adjustments Such calculated values are given in Fig 2 It can be seen that the capillary pressure in this experiment could be decreased by some 4.5 mm Hg below the isovolumetric value when the vasoconstrictor fibre activity was reflexly intensified The correctness of these indirectly deduced values for the capillary pressure alterations could be tested by measuring to what extent venous outflow pressure had to be elevated to prevent the net fluid absorption which occurred during an inactivation of the baroreceptors The principles of such a procedure are demonstrated in Fig 2 When venous outflow pressure was elevated by 5 cm H_2O (nearly 4 mm Hg) to allow for an estimation of CFC capillary pressure can be expected to rise approximately 3 to 3.3 mm Hg if the pre- to postcapillary resistance ratio is assumed to be somewhere between 4/1 and 10/1 It can then be seen (*e g* in Fig 2 B) that an elevation of the capillary pressure by 3 to 3.3 mm Hg will substantially retard but not completely eliminate the net fluid absorption occurring during a baroreceptor inactivation implying that the capillary pressure must then have been reduced by more than 3 to 3.3 mm Hg The deduced value of a 4.5 mm Hg capillary pressure decrease cannot therefore be too far from the true value On the other hand in C where the baroreceptor inhibition is less pronounced a capillary pressure rise amounting to 3 to 3.3 mm Hg will convert the net absorption to a net filtration as compared with the initial isovolumetric state The capillary pressure must thus be reduced less than 3 mm Hg and the deduced value of 1.4 mm Hg seems reasonable

From the relation between blood flow and CFC changes revealed by the five experiments in which CFC was more systematically determined (see *e g* Fig 2) the approximate shifts in capillary pressure were calculated in all experiments in this series (see *e g* Fig 1 and 3) It was regularly found that the more intense the constrictor fibre activation the more pronounced was

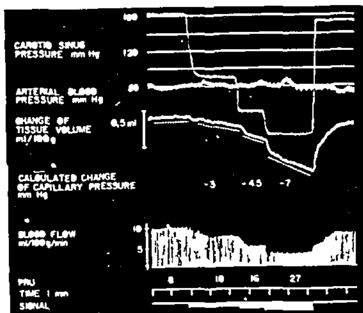


Fig 3 Cat 2 8 kg Chloralose Effects of stepwise reductions of pressure in bilaterally perfused carotid sinus regions on resistance and capacitance vessels and rate of net fluid absorption in the hindquarters Graded reductions of intrasinusal pressure are followed by stepwise increases of the rate of fluid absorption — The animal was curarized and kept under artificial ventilation Both vagal nerves were cut in the neck Arterial blood pressure was kept constant by means of a pressure compensator

the capillary pressure fall implying a progressive reflex increase in the pre to postcapillary resistance ratio One should therefore expect to find that the more the baroreceptor impulse discharge is lessened by a gradual lowering of the intrasinusal pressure the more pronounced will be the net absorption of interstitial fluid in skeletal muscle That this is indeed the case is demonstrated more clearly in Fig 3 in which the vascular reactions in the hindquarters were studied upon graded reduct ons of the intrasinusal pressure This procedure increased flow resistance and reduced regional blood volume in a step wise fashion and the resultant lowered capillary hydro tatic pressure pre sumably combined with an increased CFC produced stepwise increases of the rate of fluid absorption across the capillary membranes

If the assumption is made that the pre to postcapillary resistance ratio in the hindquarters under resting conditions amounts to 4/1 it is then possible to deduce approximately the magnitude of the reflex changes in pre and postcapillary resistance from the value for blood flow arterial and venous pressures and the calculated shift in mean capillary hydrostatic pressure The

experiment illustrated in Fig 3 can be taken as an example of this The arterial inflow pressure was here approximately 80—85 mm Hg while the venous out flow pressure was kept at 5 mm Hg which would then imply a mean capillary pressure of 20 mm in the isovolumetric state This value seems reasonable for a transcapillary pressure equilibrium, as the plasma colloid osmotic pressure in the cat is about 25 mm Hg (cf YOFFEY and COURTICE 1956 p 70) and the transcapillary colloid osmotic pressure gradient must be somewhat lower than this value due to the colloid content in the interstitial fluid When the vasoconstrictor fibre activity was reflexly increased in a stepwise fashion the capillary pressure was calculated to decline from 20 mm Hg to 17, 15 5 and 13 mm Hg respectively The precapillary resistance (arterial pressure minus mean capillary pressure divided by the blood flow) can then be calculated to have increased by 35 110 and 270 per cent respectively while at the same time the postcapillary resistance (mean capillary pressure minus venous pressure divided by the blood flow) is increased by 2 35 and 90 per cent These figures serve to illustrate quantitatively how differently the flow resistance in pre and postcapillary resistance sections is changed with reflex activations of the vasoconstrictor fibres The pre and postcapillary resistance ratio in the skeletal muscles can thus be calculated to amount to approximately 8/1 at the strongest reflex vasoconstriction shown in Fig 3

From a qualitative point of view the above experiments can be considered as representative of all those in which the baroreceptor reflex adjustments of the skeletal muscle vessels were studied There was naturally sometimes relatively marked quantitative differences between the individual experiments The findings can be summarized as follows A reduction or elimination of the baroreceptor restraint upon the vasomotor centre induces in the skeletal muscles a marked and well maintained reflex constriction of the resistance vessels a moderate relaxation of the precapillary sphincters a reflex constriction of the capacitance vessels and a net absorption of fluid from the interstitial space into the blood stream if the tissue was previously in an isovolumetric state The rate of the transcapillary fluid transfer seems to be closely related to the degree of reflex vasoconstrictor fibre activation Conversely, as a response to increased baroreceptor activity a reflex dilatation of the resistance and capacitance vessels is produced together with a net filtration of fluid from the blood into the tissue spaces

Skin Experiments on 6 animals were performed in which the baroreceptor reflex influence on the *cutaneous* vessels was studied The reactions in a skeletal muscle vascular bed were recorded simultaneously in all these experiments to allow for quantitative and qualitative comparisons of the induced reflex responses in the two tissues Fig 4 shows recordings from one of these

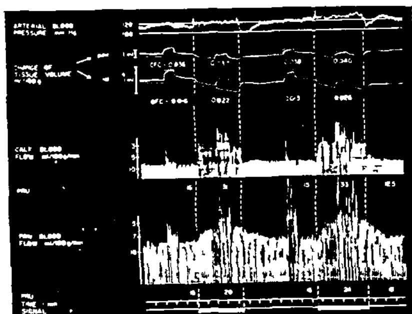


Fig 4 Cat 4 2 kg Chloralose Effects of bilateral carotid occlusion (signals) on blood flow tissue volume and capillary filtration coefficient (CFC) in skin (paw) and skeletal muscle (calf) Note the more pronounced reflex reduction of blood flow in the calf as compared with the paw Note also that CFC does not change materially in the paw with reflex augmentation of vasoconstrictor fibre activity but increases in the calf The venous outflow pressure is here elevated 10 and 20 cm H_2O in the calf and paw respectively for the CFC determination (except for the first illustrated determination where the pressure was elevated 10 cm H_2O in both tissues) — The animal was curarized and kept under artificial re p ration The vagal nerves were cut in the neck Arterial inflow pressure was kept essentially constant with an adjustable screw clamp around the abdominal aorta

experiments in which the reflex effects of a bilateral carotid occlusion upon a skin (paw with pads excluded) and skeletal muscle vascular bed (calf of the contralateral limb) were studied Blood pressure was recorded in the inferior mesenteric artery and was kept constant during the various experimental procedures by adjustments of a screw clamp placed around the abdominal aorta

The recording of paw blood flow shows that bilateral carotid occlusion (signals) induced a moderate constriction of the cutaneous resistance vessels leading to an increase of flow resistance by approximately 40 per cent When the carotid clamps were removed again the cutaneous flow resistance app roached the control value The recording of paw volume demonstrates an initial rapid phase of volume decrease when the carotid arteries were clamped implying a constriction of the capacitance vessels and an expulsion of blood

The following slower but steady volume decrease indicates that a continuous absorption of fluid into the blood across the capillary walls took place in the paw when the vasomotor fibre activity was reflexly increased The rate of this reabsorption amounted to approximately 0 14 ml/100 g tissue/min When the carotid clamps were removed the capacitance vessels again dilated and the volume record stabilized at an isovolumetric state CFC in the skin was determined during control conditions and during bilateral carotid occlusion During the control period CFC in skin was found to be 0 035—0 038 ml/min/100 g tissue/mm Hg which is about twice the value normally found in resting skeletal muscle It did not change materially upon bilateral carotid occlusion This indicates that the tone of the precapillary sphincters was not substantially affected by the increased vasoconstrictor fibre discharge

The transcapillary fluid absorption occurring in the paw when baroreceptor *reflexes were elicited indicates that a reduction of capillary hydrostatic* pressure was induced obviously due to a reflex rise of the pre to postcapillary resistance ratio One must therefore conclude that upon reflex vasoconstrictor fibre activation the precapillary resistance was increased more both relatively and absolutely than the postcapillary resistance The mean capillary hydrostatic pressure in the skin was calculated in this experiment to have been reduced by approximately 3 5 mm Hg from the isovolumetric value when the carotid arteries were occluded

The vascular adjustments in the skeletal muscle preparation were similar to those demonstrated in previous figures Furthermore from a qualitative point of view they were virtually similar to those obtained simultaneously in the skin but were quantitatively more pronounced Flow resistance in skeletal muscle was thus increased 100 per cent upon carotid occlusion as compared with 40 per cent in the skin and the calculated reduction of capillary pressure amounted to 5 mm Hg in the calf as compared with 3 5 mm Hg in the skin *However because of a greater CFC in the skin the net fluid transfer per unit* tissue weight was almost the same in the two tissues Another difference between the two vascular beds is illustrated by the fact that CFC in the skeletal muscle *increased* by approximately 40 per cent when the vasoconstrictor fibre discharge was reflexly increased but remained essentially unchanged in the skin These quantitative and partly qualitative differences in the vascular responses in skin and skeletal muscle during baroreceptor reflexes were encountered consistently in all the experiments of the type presented

Intestine The reflex adjustments of the intestin[illegible] were studied in 20 animals In 6 experiments a[illegible] was studied simultaneously The record fr[illegible] shown in Fig 5 In the left panel of th[illegible] r activity [illegible]

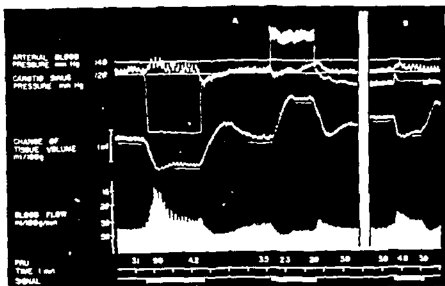


Fig 5 Cat 2 4 kg Chloralose Effects of changes in pressure and pulsation amplitude (A) and pulsation amplitude only (B) in bilaterally perfused carotid sinus regions on blood flow and tissue volume of the intestine — Note the restoration of blood flow towards normal despite continued reflex activation (or inactivation) of vasoconstrictor fibre activity The volume record demonstrates a reflex constriction and dilatation of the capacitance vessels upon lowered and raised intrasinusal pressure respectively but no net fluid transfer across the capillary membrane when steady state blood flow is established (dotted lines) — The animal was curarized atropinized and kept under artificial ventilation The vagal nerves were cut in the neck The right suprarenal was ligated the left denervated — Arterial blood pressure was kept essentially constant throughout by a pressure compensator

were produced by variations of both the mean pressure and the pulsation amplitude in the bilaterally perfused carotid sinus regions In the right panel (B) the mean intrasinusal pressure was maintained constant while the pressure pulsations were eliminated leading to a decreased baroreceptor activity The systemic blood pressure was kept essentially constant throughout the experiment by means of a blood pressure compensator The venous outflow pressure was initially adjusted so that an isovolumetric state was present

The blood flow recording shows that a reduction of the mean intra inusal pressure from the control level together with an elimination of the pressure pulsations induced a clearcut reflex constriction of the intestinal resistance vessels so that the flow resistance was initially increased approximately three times control However this constriction of the resistance vessels was not maintained during the period of reduced intrasinusal pressure but subsided fairly rapidly so that the blood flow gradually increased to reach a stedy state level only 35 per cent below that recorded during the control period

A fairly pronounced and long lasting hyperemia ensued when the intrasinusal pressure was again elevated to control level A stimulation of the carotid baroreceptors produced by elevation of intrasinusal pressure and increasing the pulsation amplitude induced a reflex increase of the intestinal blood flow reflecting a dilatation of the resistance vessels The initial effect was also now fairly pronounced decreasing PRU by 30 to 35 per cent but a partial restoration of the tone of the resistance vessels ensued so that in the steady state PRU was only some 15 per cent below the control value

The response pattern of the intestinal resistance vessels with reflex shifts in vasoconstrictor fibre activity showing first a marked initial effect which was transformed into a more moderate steady state level was a most constant and characteristic feature in all experiments in this series as long as vascular reactivity was well preserved It was especially pronounced when the vasoconstrictor fibres were more intensely activated This phenomenon is also encountered when the intestinal vasoconstrictor fibres are directly stimulated at constant rates and as it therefore must be ascribed to local mechanisms it has been called autoregulatory escape of the resistance vessels from the vasoconstrictor fibre influence (FOLKOW *et al* 1964 *a*)

The tissue volume record in Fig 5 demonstrates that a lowering of the intrasinusal pressure initiated a marked reduction in tissue volume due to an expulsion of blood from the capacitance vessels When the blood expulsion was completed the intestinal volume again increased to some extent synchronous with the autoregulatory escape of the resistance vessels This secondary slow volume gain is in all probability mainly due to a *passive* filling of the capacitance vessels consequent upon the rise of the transmural pressure in this vascular section when the precapillary resistance gradually declines However when a steady state condition with respect to flow resistance was established the tissue volume remained constant also There is thus for the intestine no indication of a further continuous diminution of tissue volume which was so regularly found in skeletal muscle and skin when the vasoconstrictor fibres were reflexly activated and there attributed to a net absorption of tissue fluid A transfer of fluid across the capillaries evidently does not take place in the intestine when the vasoconstrictor fibre activity is reflexly augmented However a clearcut net absorption of fluid always occurred when the intestinal capillary pressure was even slightly reduced by a reduction of venous outflow pressure It therefore seems reasonable to conclude that during the steady state phase of a reflex vasoconstriction the capillary pressure in the intestine was in fact not significantly altered implying that no significant shift in the pre to postcapillary resistance ratio occurred

When the intrasinusal pressure was again raised to control level the intestinal volume increased due to the reflex release of the enhanced vasoconstrictor fibre activity to the capacitance vessels The volume was often found to exceed temporarily the control level evidently due to a passive distension of the veins concomitant with the temporary decrease of the tone of the precapillary resistance vessels during the phase of reactive hyperemia When the precapillary resistance tone slowly built up again the intestinal volume approached the control level

A similar train of events occurred when the baroreceptors were stimulated above their resting activity by elevating the intrasinusal pressure and increasing the pulse amplitude There was a marked accumulation of blood in the intestine followed by a slight emptying of the veins when their transmural pressure was reduced as a consequence of the partial restoration of the tone in the precapillary resistance vessels When the tone of the resistance vessels was established at a new steady state level the volume was stabilized and remained constant There was thus no evidence for any net filtration of fluid across the capillary membranes in the intestine which was such a regular finding in the skeletal muscles The mean capillary hydrostatic pressure in the intestinal vascular bed therefore seems to be kept remarkably stable once the autoregulatory escape is completed irrespective of even wide reflex variations of constrictor fibre activity This is in agreement with the events seen in the intestine when its vasoconstrictor nerves are directly stimulated (FOLKOW *et al* 1964 *a*)

The amount of blood expelled immediately from the intestine when the intrasinusal pressure was lowered amounted in the experiment shown in Fig 5 to approximately 1 1 ml/100 g tissue as measured from the initial rapid volume reduction However following that the veins became partly filled so that during steady state conditions the net blood mobilization amounted to only 0 8 ml/100 g tissue Since venous outflow pressure and evidently also mean capillary pressure were now the same as during the control period mean transmural pressure in the capacitance compartment must also have been essentially unchanged Therefore the net blood expulsion cannot be attributed to any passive elastic recoil of the capacitance vessels but must fairly correctly reflect the degree of active reflex venoconstriction

When the baroreceptors were stimulated 1 4 ml of blood per 100 g tissue was accumulated in the intestine due to the reflex inhibition of the tonic vasoconstrictor fibre discharge to the capacitance vessels If the total blood content in the intestinal vasculature is considered to be 8—9 ml/100 g tissue during resting conditions (FOLKOW LUNDGREN and WALLENTIN 1963) it can be calculated that in the experiment shown in Fig 5 approximately 25—30

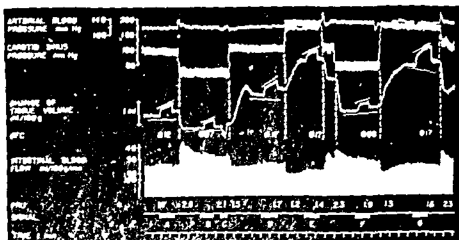


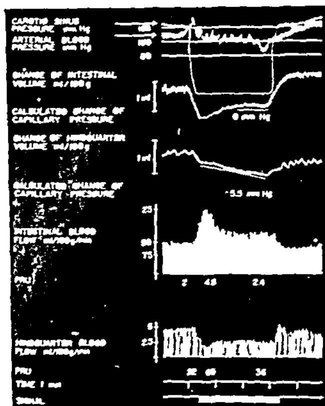
Fig. 6. Cat 3.8 kg. Chloralose. Effects of pressure variations in bilaterally perfused carotid sinus regions upon blood flow, tissue volume and capillary filtration coefficient (CFC) in the intestine. CFC is seen to decrease with reflex activation of the vasoconstrictor fibres and vice versa. Note the absence of a significant net transcapillary fluid exchange with reflex shifts in vasoconstrictor fibre activity, although a clearcut transfer of fluid across the capillary wall occurs when venous outflow pressure is increased for CFC determinations (signals) or decreased (C). — The animal was curarized, atropinized and kept under artificial respiration. The vagal nerves were cut in the neck. Arterial blood pressure was kept constant with a pressure compensator.

per cent of this volume constituted a functional blood depot, which could be mobilized by appropriate reflex shifts of the tone of the capacitance vessels.

Fig. 6 shows recordings from an experiment similar to that described in Fig. 5 with the addition that CFC was repeatedly determined (signals) to provide information concerning the response of the intestinal precapillary sphincters to baroreceptor reflexes. CFC was found to be 0.12 during resting conditions (A), a value in good agreement with that given by FOLKOW, LUNDGREN and WALLENTIN (1963). It should be noted that this value is approximately ten times greater than that of the resting skeletal muscle. This marked difference in CFC between the two tissues is probably mainly due to a far denser capillary network in the intestine, especially in the intestinal mucosa, although differences in number and/or size of capillary pores may contribute (see e.g. FOLKOW, LUNDGREN and WALLENTIN 1963). The responses of the resistance and capacitance vessels upon variations of the intrasinusal pressure were generally similar to those demonstrated in Fig. 5. The marked initial constriction and subsequent autoregulatory escape of the resistance vessels upon lowering of the intrasinusal pressure being well illustrated by the blood flow recording. The pronounced reflex shifts of tone of the capacitance vessels and the consequent changes in intestinal blood content are evident from the

tissue volume record The absence of any significant transcapillary absorption of fluid in connection with reflex increases of constrictor fibre activity or *vice versa* is also clearly shown in this experiment although marked net filtration and absorption (C) readily occurred whenever capillary pressure was changed by shifts in venous outflow pressure The present experiment further shows that CFC in the intestine *decreased* when the vasoconstrictor fibre activity was reflexly increased (B and F) and tended to increase when the constrictor fibre activity was reflexly inhibited (compare A with E and G) These reflex changes of CFC in the intestine are thus opposite in direction to those encountered in a skeletal muscle vascular bed where CFC regularly tended to *increase* when an augmented vasomotor fibre activity caused a reduction of the blood flow and *vice versa* This suggests a powerful constrictor fibre influence on those sections of the intestinal vessels which determine the number of capillaries perfused

The intestinal vascular responses in baroreceptor reflexes as illustrated in Fig 5 and 6 were typical for all experiments in this series although of course quantitative differences were seen The most characteristic feature was the *absence* of a substantial net fluid transfer across the capillary membranes in the intestine associated with baroreceptor reflex adjustments at the same time as there were pronounced reflex changes of intestinal blood flow and blood content — The marked disparity in the reflex vascular response patterns in a skeletal muscle vascular bed and an intestinal vascular bed was clearly revealed when the two tissues were studied simultaneously Such an experiment is shown in Fig 7 The vascular reactions in the two tissues were followed during reduction in the mean pressure and pulse amplitude in the bilaterally perfused carotid sinus regions The autoregulatory escape of the intestinal resistance vessels which occurred despite a maintained augmentation of vasoconstrictor fibre activity is clearly demonstrated The regional flow resistance was increased by only 20 per cent during the period of steady state blood flow conditions The constriction of the resistance vessels in the hindquarter preparation was however far better preserved and after a shortlasting initial overshoot flow resistance stabilized at a level exceeding the control value by roughly 70 per cent The volume records demonstrate an expulsion of blood from both tissues amounting to roughly 1 ml/100 g in the intestine (10—15 per cent of the calculated blood content) and 0 3 ml/100 g from the hindquarters (10—12 per cent of calculated blood content) In the hindquarter preparation a clearcut reabsorption of fluid from the interstitial spaces into the blood stream occurred indicating a reduction in mean capillary hydrostatic pressure consequent upon an increased pre to postcapillary resistance ratio There was a calculated decrease in capillary pressure of roughly



F... Cat ... kg Chloralose Effects of reduction of pressure and pulsations in the perfused right carotid sinus upon resistance and capacitance vessels and net transcapillary fluid transfer in the hindquarters and the intestine Note the marked and well sustained reflex constriction of the resistance vessels and the continuous fluid absorption in the hindquarters as contrasted to the subsiding resistance vessel response and the absence of fluid absorption in the intestine during steady state flow conditions — The animal was curarized atropinized and kept under artificial ventilation. The vagal nerves were cut in the neck Arterial blood pressure was kept constant with a pressure compensator

5.5 mm Hg In the intestine on the other hand there was no evidence of a net fluid transfer as revealed by the isovolumetric state once a steady state flow was established indicating that the pre and postcapillary resistance changes were so balanced as to keep the capillary pressure essentially unchanged

It should be mentioned that in this series of experiments a very low intrasinusal pressure was often used in order to eliminate the baroreceptor activity completely In such situations a simultaneous stimulation of the chemoreceptors is likely to occur due to interference with the blood flow through the carotid body regions (see *e.g.* DALY LAMBERTSEN and SCHWEITZER 1954) The reflex vascular adjustments evoked can then be ascribed both to a baro

receptor inhibition and to a chemoreceptor stimulation However since the normal stimulus for the baroreceptors is the distortion of the sinus wall produced by e g the pulsatile variation of the blood pressure with each heart beat a partial inactivation of the baroreceptors without any concomitant chemoreceptor stimulation can be produced by elimination of these pulsations as has been shown by FAD GREEN and NEIL (1952) This method of inducing a partial but selective baroreceptor inhibition was often used and the right panel in Fig 5 can serve as an illustration of this With this procedure the reflex vascular adjustments were found consistently to be qualitatively similar to those obtained when the intrasinusal mean pressure was lowered although less pronounced

B Chemoreceptor reflexes

a General considerations

It is uncertain whether the chemoreceptors contribute to any significant extent to the control of the circulation during normal resting conditions They are however undoubtedly of importance for eliciting compensatory cardiovascular adjustments during various emergency situations There is thus experimental evidence to indicate that the chemoreceptors are engaged in the compensatory cardiovascular adjustments following a hemorrhage COMROE (1939) and KENNEY and NEIL (1951) found that denervation of the chemoreceptors during hemorrhagic hypotension caused a further fall of blood pressure indicating that there was a tonic excitatory influence from the chemoreceptors upon the vasomotor centre during hypovolemia That the chemoreceptors are strongly activated in hemorrhage due to a decreased blood supply to the receptor areas has also been shown by an electro physiological approach (LANDGREN and NEIL 1951)

The vascular responses following chemoreceptor activation have been studied by BERNTHAL (1938) BERNTHAL and SCHWIND (1945) LOFVING (1961 a b) and DALY and SCOTT (1962) It has been established that chemoreceptor excitation induces a reflex constriction of the resistance vessels in e g skeletal muscle and intestine as well as a constriction of the capacitance vessels (KAHLER GOLDBLATT and BRAUNWALD 1962) As is the case with baroreceptor reflex mechanisms there is evidence that the various parallel coupled vascular beds are not engaged to the same extent in chemoreceptor reflexes The skeletal muscle vessels seem to be more strongly affected than e g the intestinal vessels (BERNTHAL and SCHWIND 1945) and the renal vessels (LOFVING 1961 a) at least when the chemoreceptor stimulation is not intense The detailed vascular adjustments in chemoreceptor reflexes are

however not known Since the chemoreceptors seem to be activated when there is a reduction of blood volume it would be of interest to know if they can also influence the capillary fluid transfer and extracellular fluid distribution by way of reflex adjustments of the pre- to postcapillary resistance ratio and hence be capable of contributing to the restoration of plasma volume after a blood loss

b Results

A total of 12 cats were used in this study The reactions of the skeletal muscle vessels were studied in 7 animals using the hindquarter preparation and the reactions of the intestinal vessels in 5 animals

Skeletal muscle Fig 8 presents records from an experiment in which the carotid body chemoreceptors were selectively and intermittently stimulated by shifting a constant pressure perfusion of the right carotid body region from arterial blood to venous blood the venous blood being derived from the hindquarter effluent The systemic blood pressure was maintained constant during the experiment by means of a blood pressure compensator The animal was curarized and artificial respiration was given to avoid reflex changes in respiration Stimulation of the chemoreceptors by perfusion of the carotid body region with venous blood (signals A B and D) induced a marked reduction in blood flow indicating a constriction of the resistance vessels The flow resistance thus increased approximately 2 to 2.[illegible] times the control value Upon return to arterial blood perfusion of the carotid body region flow resistance was restored to the control value The volume record demonstrates that chemoreceptor excitation induced a reflex constriction of the capacitance vessels as revealed by the initial rapid volume reduction and an absorption of fluid from the interstitial spaces into the blood stream indicated by the following slower volume decrease (dotted lines) It can be calculated that 0.[illegible] to 0.3 ml blood/100 g tissue corresponding to approximately 10 to 15 per cent of the total regional blood content was expelled from the hindquarters upon chemoreceptor excitation The amount of fluid transferred from the extravascular compartment during the chemoreceptor reflexes amounted to 0.0[illegible] to 0.0[illegible] ml/100 g tissue/min. Upon cessation of the chemoreceptor stimulation the capacitance vessels dilated and the isovolumetric state was again attained CFC was estimated during resting conditions (C) and during a period of chemoreceptor stimulation (E) It was found to increase by about 3[illegible] per cent when the reflex vasoconstriction was induced which suggests a moderate diminution of precapillary sphincter tone in connection with the reflex reduction of blood flow

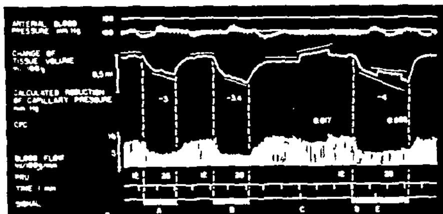


Fig 8 Cat 3 4 kg Chloralose Effects on hindquarter blood flow volume and capillary filtration coefficient (CFC) of chemoreceptor stimulation produced by perfusion of the right carotid sinus region temporarily with venous blood (signals A B and D) at an un changed pressure level (not recorded) Chemoreceptor excitation induces a reflex con tric tion of the resistance and capacitance vessels an increase of CFC (compare C and E) and a net absorption of tissue fluid across the capillary wall The animal was curarized and kept under artificial respiration The vagal nerves and the left sinus nerve were cut Arterial blood pressure was kept constant with a pressure compensator

The net absorption of fluid across the capillaries during chemoreceptor activation must as previously discussed be ascribed to a reduction of mean capillary pressure consequent upon a reflexly induced rise of the pre to post capillary resistance ratio The magnitude of the capillary pressure reduction from the isovolumetric value can be calculated from the recorded rate of net fluid absorption and the estimated CFC value The capillary pressure was thus found to have decreased some 4 mm Hg when the chemoreceptors were stimulated It can be seen that an increase in the venous pressure by 5 cm water induced for estimation of CFC (E) and corresponding to a capillary pressure rise of approximately 3 mm Hg (see page 32) can substantially retard the fluid absorption but not completely prevent it The capillary pres sure must obviously have decreased more than 3 mm Hg and the calculated value (4 mm Hg) therefore seems to be reasonable

Intestine Fig 9 illustrates records from an experiment in which the reac tions of the intestinal vessels to chemoreceptor excitation were followed The experimental procedures were similar to those in the experiment just described and illustrated in Fig 8 Perfusion of the carotid body with intestinal venous blood (signals) induced a constriction of the resistance vessels which gra dually subsided despite a continued receptor excitation The flow resistance which initially was increased by 30 to 60 per cent thus stabilized at a level exceeding the control value by only 10 to 20 per cent When the chemo

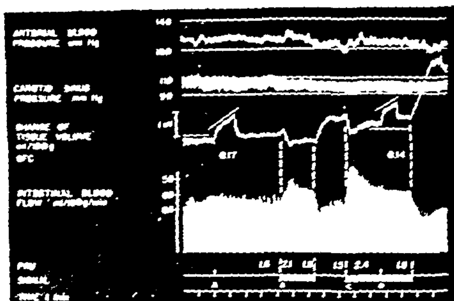


Fig. 5 Cat 2 kg Chloralose. Effects of chemoreceptor stimulation (venous perfusion of both carotid sinus regions at constant pressure, signals B and C) on intestinal blood flow, volume and capillary filtration coefficient (CFC). Chemoreceptor stimulation induces a constriction of the resistance and capacitance vessels and a reduction of CFC (compare A and D) but no net fluid transfer across the capillary membranes. — The animal was curarized, eviscerated and kept under artificial respiration. The vagal nerves were cut in the neck. Arterial blood pressure was kept constant with a pressure compensator.

receptor excitation was interrupted a considerable reactive hyperemia ensued so that the blood flow was temporarily increased to 100 ml/100 g tissue min, corresponding to a reduction in flow resistance to roughly 59 per cent of control. Synchronous with the reflex decrease of blood flow an expulsion of blood from the capacitance vessels occurred as demonstrated by the rapid downward deflection of the volume curve. This initial volume reduction was followed by a slow gain of tissue volume, in all probability due to a passive elastic distension of the capacitance vessels with the gradual decline of precapillary resistance vessel tone. However, when once a steady state blood flow was achieved tissue volume remained essentially constant. There was thus no evidence for any net exchange of fluid across the capillary membrane which suggests that the capillary pressure and hence the pre- to postcapillary resistance ratio was not significantly altered in the intestine during chemoreceptor reflex adjustment. Upon cessation of the chemoreceptor stimulation the tissue volume increased above the control level. This post-stimulatory overshoot of tissue volume was well correlated in time with the phase of reactive hyperemia. This suggests that the dilatation of the capacitance ves-

sels consequent upon release of constrictor fibre activity was enhanced by a passive venous distension when the transmural pressure in the veins was temporarily raised during the period of reactive hyperemia Estimation of CFC before (A) and during chemoreceptor excitation (D) indicates that a reduction of the capillary surface area available for fluid exchange occurred upon reflex activation of the vasoconstrictor fibres to the intestine in turn suggesting that a reflex augmentation of precapillary sphincter tone was induced

The results of the experiments in Fig 8 and 9 are qualitatively representative of all the experiments in which venous perfusion of the carotid body regions was used to elicit chemoreceptor reflexes Quite naturally quantitative differences between the individual experiments were observed One reason for this is evidently differences in P_{CO_2} P_{O_2} and pH of the venous blood used for perfusion of the carotid body region in the various experiments which will affect the extent of chemoreceptor excitation Qualitatively similar results were also obtained in the experiments in which cyanide was administered to the carotid body regions However with this type of chemoreceptor stimulation it was as a rule impossible to attain a stable level of vasoconstrictor fibre activation and a steady state as far as the vascular adjustments were concerned No detailed study of the eventually occurring fluid shifts across the capillary membranes could therefore be performed in these experiments

The results of the present series of experiments can thus be summarized as follows A chemoreceptor stimulation caused a constriction of the resistance and capacitance vessels in both skeletal muscle and intestine A net absorption of fluid across the capillary membranes occurred in the skeletal muscles due to a reflex rise of the pre to postcapillary resistance ratio and consequent reduction in capillary pressure In the intestine there was no indication of any net movement of fluid across the capillary membranes in connection with chemoreceptor reflexes indicating an essentially unchanged capillary pressure in this tissue CFC was regularly found to decrease in the intestine upon reflex augmentation of the constrictor fibre discharge while in skeletal muscle it regularly increased with increasing resistance and capacitance vessel responses

C Reflexes from heart receptors

a General considerations

The main characteristics of the cardiovascular adjustments that can be elicited from the receptors located in the walls of the heart seem to be fairly well known Elevation of the pressure in various isolated heart chambers has thus been shown to induce bradycardia blood pressure fall and dilatation of

resistance and capacitance vessels (AVIADO *et al* 1951, BAKER and KOTTEGODA 1958 AVIADO and SCHMIDT 1959 SALISBURY CROSS and RIEBEN 1960 ROSS FRAHM and BRAUNWALD 1961 *b*) From these findings the general conclusion has been drawn that the heart receptors exert an inhibitory influence upon the medullary vasomotor centre qualitatively similar to that exerted by the systemic arterial baroreceptors However quantitative information concerning the cardiovascular effects of heart receptor stimulation are more sparse mainly due to the immense technical problems involved in such an analysis especially when a complete hemodynamic isolation of various heart chambers is desired

In 1867 BEZOLD and HIRT showed that subcutaneous or intravenous injections of veratrum alkaloids induced pronounced cardiovascular and respiratory responses consisting of a bradycardia blood pressure fall and apnea The cardiovascular actions of the drug were considered to be due to a stimulation of receptors in the heart conveying their impulses through the vagal nerves since the effects were abolished after bilateral vagotomy This hypothesis was further substantiated by the work of JARISCH and coworkers (see *e g* JARISCH and RICHTER 1939 *a b*) The type of receptor stimulated by the drug and their exact location is still not fully known Electrophysiological studies have however shown that both ventricular and at least to some extent atrial receptors are excited (JARISCH and ZOTTERMAN 1948 PAINTAL 1955 NEIL and JOELS 1961) The similarities in the response pattern induced by pressure elevation in isolated heart chambers and injections of veratrum alkaloids suggest that the same types of cardiac receptors are excited by both procedures (JARISCH 1948 AVIADO 1962) The adequate stimulation of the receptors seems to be a mechanical distortion of the heart wall while the drug stimulation should rather be looked upon as an abnormal strong and unspecific type of receptor activation At any rate by intravenous injection of suitable amounts of the drug a stimulation of the heart receptors can be accomplished and the extensive operative procedures required for an isolation of the individual heart chambers can be avoided

Another aspect of the function of the heart receptors is illustrated by the investigations of HENRY GAUER and coworkers A distension of the left atrium was thus shown to induce an afferent discharge via vagal fibres (HENRY and PEARCE 1956) supposedly passing to the hypothalamus and inhibiting the release of the antidiuretic hormone leading to changes in the water excretion of the kidneys (HENRY GAUER and REEVES 1956 ZUIDEMA *et al* 1956) The existence of receptors in the right atrium by which the release of aldosterone from the adrenal glands can be affected has also been proposed (see *e g* FARRELL 1958)

b Results

In the present series of experiments the reflex effects upon various cardio vascular parameters induced by stimulation of the heart receptors have been studied in 11 cats In 8 cats the receptors were stimulated by injections of protoveratrine into the right atrium In three animals attempts were made to stimulate selectively the left atrial receptors by distension of the atrium The reflexly induced vascular adjustments in skeletal muscle or intestine were followed and in some experiments reflex changes in heart rate before and after atropinization When compared with the reflex response pattern induced from *e g* the carotid baroreceptors it was a general impression that the reflex vagal bradycardia constituted relatively the most prominent feature when heart receptors were activated whether this was done by proto veratrine injection or by distension of the left atrium This impression is based on the observation that for a given extent of reflex blood pressure fall and decrease of regional flow resistance the extent of reflex bradycardia was mostly far more pronounced when heart receptors were activated than when arterial baroreceptors were stimulated This was the case also when the efferent vagal heart fibres were blocked with atropine under which cir cumstances reflex changes in heart rate must be ascribed to shifts in accelerans fibre activity It is therefore possible that the afferent fibres from the heart receptors converge more extensively upon the efferent vagal and sympathetic fibres to the heart while those of the arterial baroreceptors seem to be more evenly distributed to all the different central neuron pools controlling the efferent sympathetic and vagal discharge to the cardiovascular system This possible differentiation of the reflex response patterns induced from the two types of cardiovascular receptors will be dealt with in more detail in a sepa rate study

Skeletal muscle In those experiments in which distensions of the left atrium were performed barely discernible cardiovascular adjustments were obtained The most prominent finding was a slight bradycardia which disappeared after section of both vagal nerves The peripheral vascular adjustments were usually insignificant

Injections of minute amounts of protoveratrine induced however very powerful reflex cardiovascular adjustments Fig 10 shows an experiment in which the vascular reactions in the hindquarters perfused at constant pres sure from a donor animal were studied when the heart receptors were stimulated by injections of 3 μg protoveratrine into the right atrium of the recipient animal (signal) The recipient animal was curarized and artificial respiration was administered to eliminate circulatory effects secondary to

4

Fig 10 Recipient cat 1 5 kg donor cat 3 9 kg Chloralose Effects of injection of 3 μg protoveratrine in the right atrium (signal) on blood pressure and blood flow and volume of the hindquarters perfused from the donor animal at constant pressure Excitation of the heart receptors induces a marked blood pressure fall a dilatation of the resistance and capacitance vessels and a net filtration of fluid from the blood into the tissue spaces — The animal was curarized atropinized and kept under artificial respiration Both carotid arteries were clamped

drug induced changes in respiration Atropine was also given to prevent the profound reflex vagal bradycardia which would have further reinforced the blood pressure fall of the recipient animal and hence elicited intense compensatory reactions from other cardiovascular receptor areas In this experiment the sinus and aortic depressor nerves were left intact but both carotid arteries were clamped partly to ensure a relatively high initial vasoconstrictor fibre activity partly to reduce the buffering effect of the carotid sinus receptors Injection of protoveratrine induced after a latency of 15 sec a marked blood pressure fall in the recipient animal and an increase in blood flow in the cross perfused hindquarters indicating a reflex decrease of regional flow resistance amounting to some 30–35 per cent The volume record shows that a reflex dilation of the capacitance vessels occurred when the heart receptors were stimulated as revealed by the initial rapid increase in tissue volume This was followed by a second slower volume gain reflecting a net filtration of fluid

from the blood into the interstitial tissues However the inhibition of vaso constrictor fibre activity caused by the stimulation of the heart receptors is not maintained constant for any length of time presumably due to compensatory reactions induced from other receptors by the marked hypotension in the recipient animal Therefore the tone of the resistance and capacitance vessels slowly built up again leading to a continuous decrease of blood flow and of tissue volume However during the first 2 minutes following the drug administration the reflexly increased blood flow was relatively stable which suggests that during this phase a fairly sustained and steady reflex inhibition of vascular tone was present The vascular reactions during this first period may thus be considered representative of those obtained by a relatively selective stimulation of the heart receptors It is seen that during this phase there was a steady slow volume increase in the skeletal muscles suggesting filtration from the blood to the interstitial space Therefore it seems justified to conclude that a stimulation of the heart receptors induces besides a dilation of resistance and capacitance vessels a reduction of the pre to post capillary resistance ratio in the skeletal muscles in a manner similar to that produced by stimulation of the arterial baroreceptors leading to a rise of capillary pressure and a filtration of fluid across the capillary membranes

Intestine Fig 11 shows an experiment in which the effect of protoveratrine upon an intestinal vascular bed was studied The intestine was perfused at an essentially constant pressure from a donor animal The recipient cat was curarized artificially ventilated and atropinized In this experiment both sinus nerves were cut as well as both aortic depressor nerves Injection of 2 μg protoveratrine in the right atrium of the recipient cat (A) induced an intense blood pressure fall and a marked dilatation of the resistance vessels of the crossperfused intestine reducing flow resistance by 40 per cent The tone of the resistance vessels as usually found in the intestine partly recovered and at steady state flow conditions the flow resistance was reduced by only about 25 per cent The volume curve shows that a dilation of the capacitance vessels also occurred when the drug was given This volume gain was followed by a slow continuous reduction of volume presumably partly due to a slow passive expulsion of blood consequent upon the gradual increase of the precapillary resistance There was however no indications that any net filtration occurred from the blood into the tissues At B in the figure the vagal nerves were cut bilaterally while the excitation of the heart receptors was still present This manoeuvre produced an immediate rise of blood pressure and an increase of tone of the intestinal resistance and capacitance vessels This may be considered to demonstrate in an exaggerated way because of the very high activity level of the heart receptors here the type of cardio-

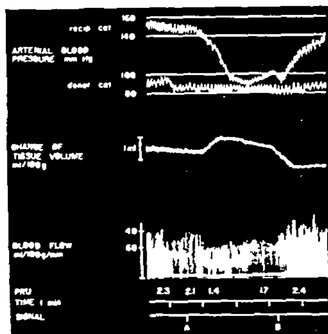


Fig 11 Recipient cat 2 4 kg donor cat 3 6 kg Chloralose Effects of injections of 2 μg protoveratrine into the right atrium (signal A) upon blood pressure and intestinal blood flow and volume of intestine perfused from a donor cat At B both vagal nerves are cut Note absence of any significant net transfer of fluid across the capillary membrane during the reflex vascular adjustments The recipient animal was curarized atropinized and kept under artificial respiration The sinus and the aortic-depressor nerves were cut bilaterally

vascular effects that will be obtained if the normally moderate tonic restraint of the heart receptors upon the vasomotor centre were suddenly eliminated *e g* by a sudden reduction of the pressure in the heart chambers as a consequence of a large blood loss

Quantitatively similar results as those illustrated in Fig 10 and 11 were obtained in all experiments in which the reactions of a skeletal muscle or intestinal vascular bed were studied upon injections of veratrum alkaloids A marked blood pressure fall and a dilatation of the resistance and capacitance vessels in both tissues were always induced In the skeletal muscle a net transfer of fluid across the capillaries from the blood into the interstitial compartments also seemed to occur due to a reduction of the pre- to post-capillary resistance ratio and the consequent reduction in capillary hydrostatic pressure In the intestine on the other hand there was no signs of a significant net movement of fluid across the capillary membranes when the heart receptors were stimulated

D Comments

The present experiments have shown that besides adjustments of tone in resistance and capacitance vessels a substantial transfer of fluid across the capillary membranes can be initiated during various cardiovascular reflex adjustments It has also been demonstrated that this transcapillary fluid exchange does not occur in all vascular beds and not to an equal extent in the various parallel coupled circuits that were studied It was found to take place primarily in the skeletal muscles and in the skin and was virtually absent in the intestine

As discussed earlier the key mechanism responsible for the transcapillary movement of fluid with the various circulatory reflex adjustments must be shifts in mean capillary hydrostatic pressure in turn dependent upon reflex alterations of the pre to postcapillary resistance ratio Thus the reabsorption of tissue fluid which always occurred in the skeletal muscles and to some extent in the skin when there was a reflex vasoconstrictor fibre activation indicates an increase of this ratio Therefore there must have been a more powerful precapillary resistance increase both in absolute and relative terms than that induced in the postcapillary resistance section In the intestine on the other hand the pre and postcapillary resistances were evidently affected to approximately the same extent during the steady state phase of cardio vascular reflex adjustments since in this tissue it was not possible to produce any net transcapillary fluid transfer There was here no fluid absorption with a reflex increase in sympathetic discharge and there was no net transfer of fluid from the blood to the tissues with inhibition of sympathetic discharge

This distinct difference with regard to the adjustments of the transcapillary exchange process between skeletal muscle and skin vascular bed on the one hand and the intestinal vascular bed on the other in the cardiovascular reflex patterns studied deserves some comments First it seems appropriate to establish whether the absence of a significant capillary fluid exchange in the intestine might be due to errors in the recording procedures Changes in the intestinal volume which have been used to record transcapillary fluid movements can also be brought about by secretion into or absorption from the intestinal lumen Further spontaneous movements of the intestine might induce intermittent volume changes due to the consequent squeezing of blood from the intestinal vascular bed It might thus be possible for instance that an intestinal volume reduction caused by a net reabsorption of fluid across the capillary membranes would be offset by a secretion of fluid or by a regional blood volume increase as a result of an inhibition of intestinal motility If so the conclusion that no transcapillary fluid exchange occurred would be

erroneous However when the intestinal vascular bed was studied atropine was regularly given and the vagal nerves were usually cut In addition in control experiments, the adrenal medullary secretion was eliminated by exclusion of the adrenals These procedures should interrupt the principal pathways for producing concomitant changes of both secretion and motility To investigate the question of secretion associated with the reflex vascular adjustments in some experiments the intestinal lumen was cannulated at each end and the cannulas led outside the plethysmograph through separate openings At no time was there any sign of a secretion flowing out through either catheter during vasoconstrictor fibre activation or inactivation, and the volume record did not show any distinctly different features whether the catheters were open to allow an secretion to escape or closed Further direct inspection of the intestine did not reveal any changes in intestinal motility which might indirectly produce volume changes in connection with vascular reflexes Moreover such a type of mechanical interference with the blood content should produce only transient volume shifts and could not therefore be responsible for the isovolumetric condition present in the steady state phase of a reflex adjustment Hence it seems most unlikely that such volume changes could occur in such a regular fashion that a possible trans capillary fluid exchange was always so exactly balanced as to be consistantly concealed Further the absence of a net capillary fluid exchange in the in testine in connection with reflex vascular adjustments cannot be ascribed to immediately counteracting tissue pressure alterations since a considerable net transfer of fluid could always be induced whenever the capillary pressure was changed by adjustments of the venous outflow pressure

Thus it must be concluded that no substantial shifts in capillary pressure can be induced reflexly in the intestine under normal circumstances This implies that the pre to postcapillary resistance ratio in this tissue is main tained essentially constant in various reflex response patterns The reason for this balanced response with respect to pre and postcapillary resistance changes during the steady state phase of reflex vascular adjustments seems to be the autoregulatory escape of the precapillary resistance vessels from the constrictor fibre influence This latter phenomenon implies that secondary adjustments of the precapillary resistance vessels occur which tend to counter act the influence of the vasoconstrictor fibres Therefore even if the intestinal vasoconstrictor responses to *e g* a carotid occlusion were fairly intense ini tially the blood flow regularly increased progressively to stabilize at a level not far below that present during control conditions In fact the stronger the initial vasoconstriction the more pronounced was this autoregulatory res toration of blood flow towards the resting value This failure to maintain a

stable reflex constriction of the intestinal resistance vessels was not due primarily to any progressive decline of sympathetic impulse discharge from the vasomotor centre since simultaneously recorded reflex vasoconstriction in skeletal muscles was well sustained (see *e g* Fig 7) Moreover the 'autoregulatory escape occurs just the same when the intestinal vasoconstrictor fibres are stimulated directly at constant rates (FOLKOW *et al* 1964 *a*) Such findings as well as the observation that the reflex constriction of the intestinal precapillary sphincters and capacitance vessels is reasonably well upheld makes it clear that the subsiding constriction of the intestinal resistance vessels is primarily due to local factors The true nature of such an autoregulatory escape essentially confined to the precapillary resistance vessels is still unknown and is not revealed by the present study (for details see FOLKOW *et al* 1964 *a b*) It is possible that at least some of the components involved are related to those engaged in producing autoregulation of intestinal blood flow upon primary reductions of the perfusion pressure (JOHNSON 1960 JOHNSON and HANSSON 1962) Whatever the cause of the autoregulatory escape might be it has the functional consequence that the precapillary resistance response soon decreases until the initial pre to post-capillary resistance ratio is again established and in the steady state phase no essential change in capillary pressure is obtained It should however be mentioned that during the shortlasting initial peak of vasoconstrictor response the pre to postcapillary resistance ratio is in all probability increased with a resultant transient fall in intestinal capillary pressure and probably with an also transient movement of fluid from the extravascular space into the blood stream This can be deduced from the passive elastic recoil of the veins ocurring during this initial phase of the constrictor response which suggests that there must then be a temporary pressure drop at the capillary level

In skeletal muscles and in skin on the other hand the reflexly induced constrictions of the precapillary resistance vessels were normally well maintained and when a steady state flow was established a clear cut increase of the pre to postcapillary resistance ratio was always present to judge from the continuous reabsorption of tissue fluid in these two tissues upon *e g* a reduction of baroreceptor activity

The present study has shown also that the size of the filtering area of the capillary membrane in muscle and intestine can be significantly altered in the various reflex vascular adjustments but in opposite directions in the two tissues This was revealed by the changes in CFC which presumably reflect changes in tone of the precapillary sphincters (FOLKOW and MELLANDER 1960 COBBOLD *et al* 1963 FOLKOW *et al* 1964 b) It was found con

sistently that CFC was *increased* in the steady state phase of a reflex vaso constriction in skeletal muscles indicating a diminution in the tone of the precapillary sphincters This might at first sight seem surprising since there is evidence to indicate that the sphincters too are exposed at least to some extent to the excitatory influence of the vasoconstrictor fibres (RENKIN and ROSELL 1962) The observations in this study however are in agreement with those made by COBBOLD *et al* (1963) who also suggested a reasonable explana tion for this apparent discrepancy The reduction in blood flow, due to vaso constrictor fibre activation will promote the regional accumulation of vaso dilator metabolites A competition is then created between nervous constrictor and metabolic dilator factors and so far as the precapillary sphincters are concerned the local influence appears to dominate The consequence of this compensatory reduction in sphincter tone will be that in the muscles, the reduced blood flow is now distributed to a larger number of capillaries This will increase the surface area and prolong the time available for exchange and decrease the average diffusion distance to the tissue cells A more ade quate nutritional supply of the muscle cells is thus automatically obtained for any given blood flow With regard to the net fluid exchange it means that a bigger absorption can be accomplished for any given decrease in capil lary hydrostatic pressure

In the intestine on the contrary it was found regularly that CFC *decreased* upon reflex activation of the vasoconstrictor fibres This would seem to reflect regional differences both in vascular organization and constrictor fibre distri bution to the functionally different vascular sections of the two tissues (cf FOLKOW *et al* 1964 *b*) Few details are known at present in this respect and it should only be mentioned here that CFC in the intestine was never reduced reflexly to such a low figure that this in itself could explain the absence of transcapillary fluid absorption when the vasoconstrictor fibres were activated

The findings in this study concerning the adjustments of the resistance and capacitance vessels in the various cardiovascular reflex mechanisms are from a qualitative point of view in accordance with those reported in earlier studies (For ref see pp 26 43 48) Both the resistance and capacitance vessels were found to constrict in all three tissues studied when the baroreceptor and presumably the heart receptor activity was decreased or the chemo receptor activity was increased while the opposite procedures induced a dilatation of both of these vascular sections A comparison of the magnitude of the reflex responses of the resistance vessels revealed however that the resistance vessels of the skeletal muscles were more powerfully affected than those of the intestine or the skin (cf LOFVING 1961 *a b*) Such a differentia tion of the regional vascular responses may depend on several factors The

different neuron pools in the bulbar vasomotor center controlling the vaso constrictor discharge to the various circuits may for instance exhibit some what different excitation thresholds or be exposed to a varying extent of afferent fibre convergence so that a given excitatory influence affects some of the neuron pools more than others (FOLKOW JOHANSSON and LOFVING 1961) It is also possible that the extent of constrictor fibre supply varies considerably among the various vascular beds Further the sensitivity of the vascular smooth muscles to the transmittor substance and/or to local auto regulatory mechanisms might differ considerably modifying the neuro genically induced changes of vascular tone This latter mechanism seems to be the essential explanation for the relatively small reflex responses of the intestinal resistance vessels as compared with those in the skeletal muscles since direct uniform stimulations of the intestinal vasoconstrictor fibres mimic the reflexly induced responses (FOLKOW *et al* 1964 *a*) The skin vessels however are known to respond even more vigorously than the skeletal mu cle vessels when their vasoconstrictor fibres are *directly* stimulated at the same rates (CELANDER and FOLKOW 1953 CELANDER 1954) while upon reflex vasoconstriction the reverse is true It therefore seems justified to conclude that the cardiovascular receptors engage the vasoconstrictor fibres to the skin less extensively than those to the skeletal muscles except perhaps when both are exposed to a very strong excitatory drive The reason might simply be that the skin vessels are primarily engaged in the homeostatic regulation of body temperature which in most situations overrules adjust ments induced by cardiovascular control mechanisms

The adjustments of the capacitance vessels in baroreceptor reflexes eva luated quantitatively by the relative amount of blood translocated from the various organs seem to be if anything more pronounced in the intestine than *e g* a hindquarter preparation A probable reason for this is that a rela tively large fraction of the blood content in the hindquarters is contained in the skin vessels (MELLANDER 1960) which as suggested above are relatively less engaged by the cardiovascular reflexes The result will then be that a relatively smaller portion of the total hindquarter blood volume is mobilized when vasoconstrictor fibres are reflexly activated by a lowering of the baro receptor activity

Similar comparisons of the extent of the reflex vascular responses in various parallel coupled vascular beds were not performed when the chemoreceptors or the heart receptors were activated as in these experiments only a single vascular bed was studied at a time The relatively moderate vascular adjust ments often obtained in the intestine upon chemoreceptor stimulation as compared with those in the skeletal muscles can at least partly be ascribed

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sites in the heart and adjacent vessels with a simultaneous recording of the appropriate vascular parameters which so far has not been possible In the present study, no definite differences were found between the engagement of capacitance and resistance vessels in reflexes elicited from heart receptors or from any other receptor areas studied It should be realized however that the type of heart receptor stimulation used here (mainly by veratrum injections) seems to affect all heart receptors atrial as well as ventricular and any quantitative dissimilarities in the balance of the reflex response pattern elicited from these different receptors will then easily be concealed Moreover the drug administration must also be considered to cause a very intense receptor stimulation with such a drastic inhibition of sympathetic discharge rate that a possibly existing moderate differentiation in their inhibitory influence on central sympathetic structures becomes entirely masked

CHAPTER IV

Effects of hemorrhage upon net transcapillary fluid exchange

a General considerations

The most appropriate way to evaluate the potency of the various mechanisms aimed at maintaining the fluid content in the cardiovascular system reasonably constant is to examine what happens to the blood volume after it has been suddenly reduced by e g a hemorrhage As mentioned in Chapter I an abundance of experiments of this type have been performed The majority of these studies have shown that a fairly rapid gain of plasma volume then occurs accomplished by an entrance of protein poor fluid into the circulation across the capillary membranes However little is known concerning the exact mechanisms responsible for this transcapillary fluid movement Even if the reduction in arterial and venous pressures following a significant hemorrhage will promote the absorption of fluid from the tissue spaces as has been suggested it is likely that other more specific adjustments are brought into play A restoration of plasma volume thus takes place also when the hemorrhage is of such a small magnitude that the arterial and venous are not changed to any significant degree (MEEK and EYSTER 1921 CHIEN 1958) On the other hand the posthemorrhagic fluid replacement is definitely impaired after a sympathectomy (CHIEN 1958) This latter observation indicates that appropriate neurogenic adjustments of the cardiovascular system can contribute significantly to the mobilization of tissue fluid into the circulation in hypovolemic states (GREGERSEN and RAWSON 1959 SJÖSTRAND 1952) though as mentioned the exact nature of these adjustments has thus far not been investigated in any detail

The experiments presented in Chapter III indicate however that the organism possesses potent regulatory mechanisms by which not only flow resistance and vascular capacity can be adjusted but also the distribution of the extracellular fluid between the extra and intravascular compartments By reflex resettings of the pre to postcapillary resistance ratio in appropriate vascular circuits large volumes of fluid can be readily exchanged between the two compartments across the capillary membranes It seems reasonable that these regulatory devices are utilized when a sudden disturbance in the balance between vascular capacity and content is encountered and that they

then are capable of inducing an increase of the plasma volume at the expense of the interstitial fluid reservoir

The series of experiments described in this chapter was designed primarily to provide information whether and approximately to what extent the fluid mobilization across the capillary membranes in various circuits can be affected by graded moderate reductions of the blood volume In the majority of these experiments the arterial inflow and venous outflow pressures were kept constant to allow a more direct evaluation of the degree of the reflex changes of the pre to postcapillary resistance ratio induced The results of these experiments are described under (*1*) in the results

In other experiments the arterial inflow pressure was allowed to vary in accordance with the changes in systemic blood pressure consequent upon the hemorrhage In these experiments the transcapillary fluid transfer was followed before and after acute denervation of the vascular bed studied to provide information as to what extent the reduced inflow pressure *per se* could affect the fluid mobilization across the capillary walls The results of these experiments are presented in (2) The experiments presented in (*1*) and (2) were performed with all cardiovascular receptors intact

In still another group of animals the vascular adjustments and fluid mobilization upon standardized bleedings were followed before and after elimination of one or both of the main cardiovascular receptor sites which was accomplished by cutting the vagal aortic depressor nerves and/or the sinus nerves bilaterally These studies which are described under (3) in the results were intended to elucidate whether the capability of the homeostatic control systems to induce appropriate peripheral vascular adjustments during a hemorrhage diminished largely in proportion to the reduced number of sensing elements or whether the reflex cardiovascular response to a hemorrhage was in any way changed in its pattern when the influence of the various receptor stations was eliminated

The hemorrhages were throughout this study performed by fairly rapid (0 5—2 ml/sec) withdrawals of blood from a cannulated artery usually the brachial or left renal artery into a syringe

b Results

1 A total of 27 cats was used in this series of experiments The reactions in a skeletal muscle vascular bed was studied in 21 animals of which the intestinal vessels were studied simultaneously in 4 and the skin vessels in 6 experiments The intestinal vascular bed alone was studied in 6 cats

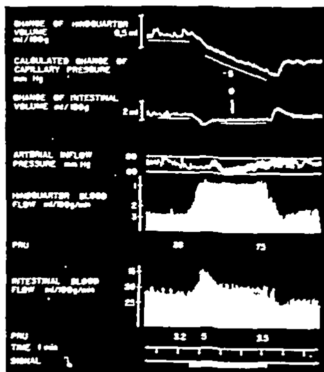


Fig 12 Cat 3 1 kg Chloralose Effect of hemorrhage (10 ml/kg bodyweight) upon blood flow and tissue volume of the hindquarters and the intestine Note the marked and sustained reduction in blood flow and the continuous absorption of tissue fluid across the capillary walls in the hindquarters as contrasted with the small blood flow reduction and the absence of fluid absorption in the intestine during steady state blood flow conditions — The animal was curarized atropinized and kept under artificial respiration — Arterial inflow pressure to both organs was kept essentially constant during the procedure by adjustments of a screw-clamp around the aorta just below the diaphragm

Fig 12 shows recordings from an experiment in which the reactions of a skeletal muscle vascular bed (hindquarters) and an intestinal vascular bed were studied simultaneously upon a hemorrhage comprising 10 ml/kg body weight corresponding to 15 per cent of total blood volume which has been assumed to comprise approximately 70 ml/kg bodyweight (cf HAMLIN and GREGERSEN 1939) By means of a screw clamp placed around the aorta just below the diaphragm the arterial inflow pressure to both organs was reduced prior to the hemorrhage to a level expected to be reached during the hypo volemia When the bleeding was then induced the clamp was adjusted so as to maintain the inflow pressure essentially constant The venous outflow pressure of each vascular bed was adjusted so as to establish an isovolumetric state in each organ The blood flow recordings show that when the blood is

withdrawn a constriction of the resistance vessels occurs in both organs In the hindquarters flow resistance was increased 2 5 times control and was well maintained at this level throughout the hypovolemic period In the intestine on the other hand the initial increase of flow resistance amounted only to some 50 per cent This constriction of the intestinal resistance vessels was moreover not sustained but subsided so that in the steady state phase of the reflex adjustment the flow resistance was only 10 per cent above the control value

The tissue volume recordings demonstrate that a reflex constriction of the capacitance vessels was induced in both skeletal muscle and intestine resulting in an expulsion of blood amounting to 0 3 ml/100 g from the hindquarters and approximately 1 ml/100 g from the intestine In the hindquarters a rapid absorption of fluid across the capillary membranes was also induced as indicated by the continuous decrease of tissue volume during steady state blood flow conditions (dotted line) The rate of fluid absorbtion into the blood stream from the extravascular space of the hindquarters amounted in this experiment to 0 1 ml/min In the intestine no such slow phase of volume decrease is seen On the contrary once the intestinal blood flow was stabilized at a slightly reduced level and the initial volume changes were completed an isovolumetric state was again attained These findings suggest that a rise of the pre to postcapillary resistance ratio and a fall of the capillary pressure here amounting to 5 mm Hg was reflexly induced in the skeletal muscles upon a blood withdrawal while no substantial change in capillary pressure occurred in the intestine

When the shed blood was retransfused there was a short period of hyperemia in both vascular circuits after which the tone of the resistance vessels slowly returned towards the control levels There was also a dilatation of the capacitance vessels and an accumulation of blood in both vascular circuits in an amount largely corresponding to that expelled during hemorrhage The intestinal tissue volume thus returned to its original level after a short overshoot which is presumably due to a passive filling of the veins consequent to the reduced precapillary resistance vessel tone during the reactive hyperemia The hindquarter volume however remained somewhat reduced by an amount which was approximately equivalent to the amount of extravascular fluid removed from the organ during the period of hypovolemia

In three experiments the capillary filtration coefficient (CFC) was more systematically estimated in skeletal muscle and intestine before and during hemorrhage to provide information as to whether a reflex shift in the size of the perfused capillary surface area and hence of precapillary sphincter tone occurred These experiments showed that CFC regularly *increased* in the ske

letal muscles when blood flow was reflexly decreased A reduction of the skeletal muscle blood flow to half the control yielded a 10—20 per cent increase of CFC and when the blood flow was reflexly reduced to half to one fifth of the control, CFC increased by 20—50 per cent In the intestine on the other hand CFC was always found to *decrease* by 10—20 per cent when the animal was bled

Fig 13 shows the effects of hemorrhage comprising 7 ml/kg bodyweight (approximately 10 per cent of the total blood volume) upon a skin (paw) and a skeletal muscle (calf) vascular bed The sudden blood withdrawal induced a constriction of the resistance vessels in both tissues as demonstrated by the blood flow recordings and the calculated values for flow resistance in the two vascular circuits It is seen that the reflex increase in flow resistance was more pronounced in the skeletal muscles (approximately 40 per cent increase) than in the skin (about 10 per cent increase) The constriction of the re istance vessels was however well preserved in both vascular beds as long as the hypovolemia was maintained — The volume recordings demonstrate that a constriction of the capacitance vessels leading to a small expulsion of blood occurred in both vascular circuits as well as a continuous absorption of fluid into the blood stream across the capillaries during the entire hypovolemic period (signal) The rate of fluid absorption was comparable in magnitude in the two tissues when calculated per unit weight of tissue and was approximately 0 1 ml/min/100 g — In this experiment the capillary filtration coefficient was determined in both tissues during the hypovolemic period to permit an estimation of the reflex shifts in capillary hydrostatic pressure from the recorded rate of net fluid absorption The capillary pressure in the skin vascular bed was accordingly calculated to be reflexly lowered by 2 mm Hg while in the skeletal muscle the corresponding value was 5 mm Hg This difference in capillary pressure change between the two vascular beds is also evident from the effects of the venous pressure rise of 5 cm H_2O induced for the CFC estimation on the rate and direction of the fluid transfer A venous pressure elevation of this magnitude which can be expected to elevate mean capillary pressure about 3 mm Hg provided the pre to post-capillary resistance ratio is 4/1 in both tissues can be seen to convert the net absorption of fluid in the skin to a net filtration which indicates that the capillary pressure was here reduced less than 3 mm Hg from the isovolumetric value In the skeletal muscle vascular bed on the other hand the elevation of capillary pressure by 3 mm Hg reduced but did not completely eliminate the continuous reabsorption of fluid which thus demonstrates that the capillary pressure was here decreased more than 3 mm Hg When the shed blood was retransfused the tone of the re istance and capacitance vessels

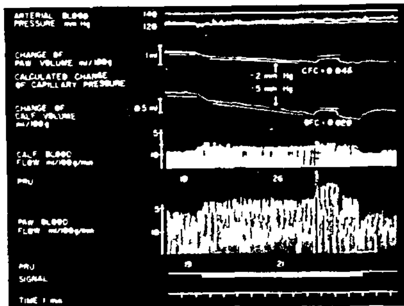


Fig 13 Cat 4 0 kg Chloralose Effects of hemorrhage (7 ml/kg bodyweight) on the resistance and capacitance vessels and net transcapillary fluid exchange in skin (paw) and skeletal muscle (calf) Note the more marked resistance vessel response in the calf than in the paw — The animal was curarized and kept under articial respiration Arterial inflow pressure was maintained essentially constant during the experiment by adjustments of a srew-clamp around the aorta.

returned to control and isovolumetric states were again attained A net reduction in the tissue volume was however encountered in both tissues largely equivalent to the amount of interstitial fluid removed during the hypovolemic state

The experiments in this series as illustrated in the figures demonstrate that even fairly small blood losses were able to produce distinct changes in regional blood flow and blood content and a significant transcapillary fluid exchange in skeletal muscle and skin Thus a blood loss comprising 5 ml/kg body weight corresponding to less than 10 per cent of the total blood volume regularly evoked unequivocal peripheral vascular responses Clearcut compensatory adjustments as a rule most pronounced in the skeletal muscles could be recognized in many experiments when only 1—2 ml blood/kg body weight or some 2—3 per cent of the assumed total blood volume was shed An experiment demonstrating this phenomenon is shown in Fig 14 The hindquarters of an animal were perfused at an essentially constant pressure head from a donor cat When 2 ml of blood per kg body weight were removed from the recipient animal (A) there was a small but definite constriction of

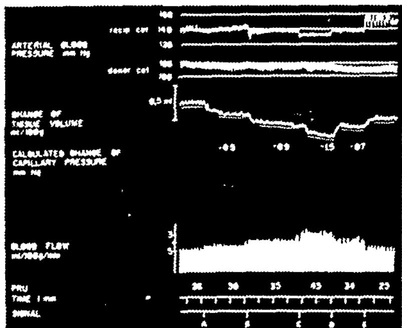


Fig 14 Recipient cat 2 kg donor cat 3 0 kg Chloralose Effects of stepwise reductions of blood volume (2 ml blood/kg bodyweight is withdrawn at A B and C) and retransfusion of the shed blood (D and F) upon resistance and capacitance vessels and net fluid transcapillary exchange in the hindquarters (perfused at constant pressure from a donor animal) Note that a hemorrhage comprising 2 ml/kg bodyweight does not affect the mean arterial pressure noticeably but initiates clearcut vascular adjustments — The recipient animal was curarized and kept under artificial respiration

the resistance and capacitance vessels of the hindquarters and a steady absorption of fluid from the interstitial space These adjustments took place without any detectable change in arterial blood pressure in the bled animal which seems to indicate that this blood volume reduction could be fully compensated for by appropriate reflex shifts in heart efficiency and peripheral vascular tone By stepwise reductions of the blood volume by 4 and 6 *ml/kg* body weight (B and C respectively) the vascular adjustments became more and more pronounced and the rate of fluid absorption increased proportionally A small drop in blood pressure in the recipient cat now also appeared indicating that a blood loss of that magnitude could not be completely compensated for When the shed blood was retransfused in two portions (D and F) the compensatory vascular adjustments went in the opposite direction i e the resistance and capacitance vessels dilated again to reach the control value when all the shed blood had been returned to the animal (F) The rate of fluid absorption was reduced in a stepwise fashion and an isovolumetric state was again obtained when the retransfusion had been completed

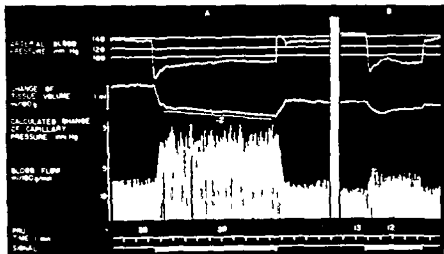


Fig 15 Cat 3 kg Chloralose Effects of hemorrhage (10 ml/kg bodyweight) on resistance and capacitance vessels and net transcapillary fluid transfer in the hindquarters before (A) and after (B) cutting the regional sympathetic vasoconstrictor fibres Note the absence of fluid absorption upon blood loss after denervation despite a marked fall in arterial blood pressure — The animal was curarized and kept under artificial respiration

The experiments illustrated here can be considered from a qualitative point of view as representative of all in this series Thus even minute blood losses produced a constriction of resistance and capacitance vessels in all the organs studied though being regularly most pronounced in the skeletal muscles Further a clearcut net fluid absorption across the capillaries occurred in skeletal muscle and skin while a significant extravascular fluid mobilization was never observed in the intestine

2 In five animals the effects of a standardized hemorrhage was studied in a skeletal muscle vascular bed before and after section of the regional vaso constrictor fibres Fig 15 shows one of these experiments in which a hind quarter preparation was used Approximately 10 ml blood/kg bodyweight was withdrawn and the hypovolemia was then maintained for a fairly long time period (10 min signal) Between the two panels in the figure the hindquarter vessels were acutely denervated by cutting the sympathetic trunks in the lumbar region The records demonstrate that as long as the vasomotor fibre supply was preserved a blood loss caused a constriction of the resistance and capacitance vessels and a continuous absorption of fluid across the capillary membranes Moreover this absorption proceeded at a virtually constant rate for the whole period of hypovolemia i e 10 min In this time period 1 8 ml

fluid was mobilized from the hindquarters It can be calculated that the capillary pressure must have been decreased by roughly 2 mm Hg to account for this rate of fluid absorption If the same capillary pressure reduction and fluid absorption were to occur in all skeletal muscles which are assumed to comprise 45 per cent of the total bodyweight (SKELTON 1927) then approximately 25 per cent of the shed blood volume would have been replaced within the 10 min period of hypovolemia This would imply that some 3—5 per cent of the extracellular fluid of the skeletal muscle mass should have been transferred to the intravascular compartment

After retransfusion of the blood vascular tone returned to the original level and an isovolumetric state was again attained However the tissue volume remained reduced to an extent corresponding to the amount of fluid absorbed during the hypovolemia This experiment shows among other things that a fairly large amount of fluid can be mobilized from skeletal muscle without causing such a marked decrease in tissue pressure or increase in tissue colloid osmotic pressure as to counterbalance the capillary pressure decrease

In the second panel the hindquarter blood vessels were acutely denervated The blood flow increased considerably upon this procedure due to a dilation of the resistance vessels There was also a rapid gain of tissue volume (not shown in the figure) owing to a dilatation of the capacitance vessels A slight and transient net filtration of fluid from the blood across the capillary walls as a result of a reduced pre to postcapillary resistance ratio also occurred after denervation The venous outflow pressure was therefore lowered somewhat so that an isovolumetric state was again attained — An acute blood withdrawal of the same magnitude as in the left panel (10 ml/kg bodyweight) led to a blood pressure drop of a comparable magnitude There was then however no measurable increase in the flow resistance in the hindquarters but if anything a slight dilatation of the resistance vessels The reduction of tissue volume ensuing upon the hemorrhage was probably due to a passive emptying of the veins owing to a slight reduction in transmural pressure consequent upon the fall of the arterial pressure When this passive blood expulsion is completed the volume remains essentially constant and there is no evidence for any significant net transcapillary fluid transfer The isovolumetric state was on the whole preserved throughout the period of hypovolemia — When the shed blood was retransfused the blood flow increased commensurate with the increase in blood pressure and a slight presumably passive increase of blood content occurred also — This experiment which was representative of all five in this series demonstrates clearly that a moderate arterial blood pressure fall following a hemorrhage is in itself not capable

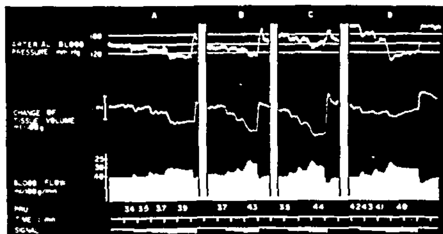


Fig 16 Cat 2 9 kg Chloralose Effects of stepwise withdrawals of 3 ml blood/kg body weight (signals) on blood flow and tissue volume of the intestine before and after elimination of the main cardiovascular receptor areas. In A all receptors are intact in B the right and in C also the left vagus nerve is cut and finally in D both sinus nerves as well — Note that the vascular adjustments to hemorrhage (in terms of e g PRU changes) are somewhat more pronounced after vagotomy but virtually absent when all receptors are eliminated — The animal was curarized atropinized and kept under artificial respiration

of inducing any significant change in capillary pressure and mobilization of extravascular fluid This is probably mainly due to the autoregulation of the precapillary resistance vessels which is seen in skeletal muscles when blood pressure is changed and central nervous influences abolished (e g FOLKOW and ÖBERG 1961) This phenomenon will be discussed in more detail below

3 In five experiments the extent of the peripheral vascular adjustments to standardized hemorrhage were measured in more detail before and after elimination of the main cardiovascular receptor areas In three experiments the hindquarter preparation was used in the remainder the intestine Fig 16 shows recordings from one of these latter experiments Stepwise withdrawals of blood comprising 3 ml per kg bodyweight each time (signals) were performed with all receptors intact (A) after cutting the right (B) and also the left vagal aortic depressor nerves (C) and finally when both sinus nerves had been severed (D) — Panel A demonstrates that a blood withdrawal corresponding to 3 ml/kg bodyweight induced a slight blood pressure fall a small but discernible constriction of the intestinal resistance vessels and a constriction of the capacitance vessels leading to an expulsion of approximately 0 ° ml of blood — With further blood volume reductions the blood

pressure fall became more pronounced and a progressive but still moderate increase in flow resistance and decrease in regional blood content occurred Thus when a total of 9 ml of blood per kg bodyweight were shed the peripheral resistance was increased by approximately 10—15 per cent during the steady state of reflex vascular adjustments while the regional blood volume was then diminished by approximately 0 8 ml There was however no evidence for a net fluid transfer across the capillary membranes during the hypovolemia but the isovolumetric state was well maintained throughout the hypovolemic period This experiment should thus be compared with that illustrated in Fig 14 in which stepwise increases in the rate of transcapillary fluid reabsorption occurred in a skeletal muscle when the blood volume was reduced in a stepwise fashion as in the present experiment

The recordings illustrated in B and C in Fig 16 demonstrate that even after section of one or both vagal aortic depressor nerves appropriate compensatory adjustments of the cardiovascular system can indeed be induced upon a sudden hemorrhage The fall in the arterial blood pressure is not more pronounced and the adjustments of the intestinal resistance and capacitance vessels were if anything more marked than the responses obtained when all receptors were intact A withdrawal of in total 9 ml blood per kg bodyweight produced at that point an increase of flow resistance by roughly 25—30 per cent and an expulsion of 1 1—1 2 ml of blood (C) — However when the sinus nerves as well were severed practically no compensatory reactions seemed to take place (D in Fig 16) The blood pressure fell markedly the flow resistance did not change materially and there was no indication of any active constriction of the capacitance vessels The small volume reductions which now followed upon the hemorrhage were in all probability due to a passive emptying of the veins due to the slight reduction in the transmural pressure caused by the fall in systemic pressure and the at least initial more marked reduction in the blood flow When however the precapillary vessels of the intestine gradually dilated as a consequence of the autoregulation to pressure changes (see e g JOHNSON and HANSON 1962) the veins were again passively filled

This experiment can be considered representative of all in this series Thus it was found regularly that an elimination of the receptors in the heart and aortic areas did not bring about any significant diminution in the potency of the reflex mechanisms responsible for the compensatory vascular adjustments in hemorrhage It was on the contrary often found that the peripheral vascular responses were if anything more pronounced after section of the vagal aortic depressor nerves This at first sight puzzling phenomenon will be discussed in more detail below

c Comments

Earlier studies have provided convincing evidence that a mobilization of extravascular fluid into the blood stream takes place in hemorrhage (for references see Chapter I) It has generally been assumed that the fall in arterial and venous pressures following a substantial blood loss is the essential mechanism responsible for the decreased capillary pressure and the ensuing fluid absorption across the capillaries If this were the case no significant restoration of plasma volume would be possible until the homeostatic control mechanisms which supposedly serve to keep the arterial and venous pressures essentially constant begin to fail There is however as mentioned earlier some evidence to indicate that a replacement of the intravascular fluid after hemorrhage occurs despite essentially unchanged arterial and venous pressures This suggests that other more specific mechanisms are also involved in the restoration of plasma volume and that these mechanisms at least to some extent are dependent upon an intact sympathetic nervous system (see e g CHIEN 1958)

The present experiments in which the absorption of fluid across the capillaries in different tissues has been followed after a blood loss are in general accordance with these earlier studies They also demonstrate the importance of adequate reflex adjustments of vascular tone for the fluid mobilization Thus by means of reflex increases in the pre to postcapillary resistance ratio a substantial absorption of tissue fluid can take place after a blood loss despite constant arterial and venous pressures This finding suggests that a restoration of plasma volume can be initiated by the reflex mechanisms responsible for the compensatory adjustments of the circulation in hypovolemic states and that it might in fact be looked upon as a most important component of these reflex adjustments rather than being merely a consequence of a relative failure of the homeostatic control devices

There is no doubt that a fall in central *venous* pressure will reduce capillary pressure and hence cause a fluid absorption at the cost of a reduced filling pressure of the heart On the other hand the present experiments suggest that a fall in the *arterial* pressure is in itself not capable of affecting the capillary pressure to any significant extent unless the pressure drop is profound The reason for this relative constancy of the capillary pressure despite wide variations in arterial pressure seems to be the pronounced autoregulation of the precapillary resistance vessels (FOLKOW and ÖBERG 1961 JOHNSON and HANSON 1962) This autoregulation appears to be due to the combined effect of the reduced transmural pressure and the relative accumulation of metabolites in connection with the pressure induced reduction in blood

flow Both of these factors tend to depress the inherent myogenic activity of the smooth muscles in the precapillary resistance vessels causing them to relax (cf FOLKOW 1962) This will lower the pre to postcapillary resistance ratio and hence cancel out the expected changes in capillary pressure upon a fall in the systemic pressure Not until the precapillary resistance vessels are maximally dilated will a reduction in arterial pressure cause a material decrease in capillary pressure

The fluid absorption across the capillaries after a hemorrhage is to judge from the present experiments not a generalized process, but takes place predominantly in the skeletal muscles and in the skin but apparently not in the intestine This implies that significant reflex alterations in the pre to postcapillary resistance ratio with blood loss occur in the skeletal muscles and the skin while in the intestine this ratio evidently remains essentially unchanged The reason for this latter phenomenon seems to be as was seen also when graded baroreceptor reflex adjustments were induced (see Chapter III) the autoregulatory escape of the intestinal precapillary resistance vessels that occurs soon after the onset of a vasoconstrictor fibre activation It has the consequence that in the steady state phase of the reflex vasoconstriction the precapillary resistance is only moderately increased while at the same time the increase in the postcapillary resistance seems to be well maintained (see FOLKOW *et al* 1964 *a*) — In the skeletal muscles on the other hand a more extensive and well maintained reflex constriction of the precapillary resistance vessels occurred with hemorrhage to judge from the sustained increase in the flow resistance and in the pre to postcapillary resistance ratio in this tissue In the skin a similar well maintained reflex increase of flow resistance and rise of the pre to postcapillary resistance ratio was observed but the extent of the reflex cutaneous vascular adjustments was always less pronounced than in the skeletal muscles

Thus a quantitatively differentiated reflex vascular response similar to that found in *e g* baroreceptor reflexes (see Chapter III) was evidently induced by the fairly small reductions in blood volume produced in the present experiments The relatively weak responses of the skin vessels are probably due to a less extensive engagement of their constrictor fibres in cardiovascular homeostatic reflex mechanisms while the comparatively small increase in intestinal flow resistance during the steady state phase of vascular adjustments seems to be due to local factors *i e* the autoregulatory escape of the intestinal precapillary resistance vessels Several previous studies have likewise shown that the flow resistance in the intestine is often very little changed after a hemorrhage (REYNELL *et al* 1955 SAPIRSTEIN BUCKLEY and OGDEN 1955 SELKURT and BRECHER 1956 FRIEDMAN 1961) Still other investigators

however have found a substantial increase in mesenteric flow resistance after a blood loss (see *e g* GRECG 1962 ABEL and MURPHY 1962) The reason for this discrepancy is not known but possibly it is due to the susceptibility of the autoregulatory escape mechanism to various deteriorating procedures Thus in the present experiments these locally induced reactions of the precapillary resistance vessels tended to vanish in those occasional cases in which the intestinal preparation deteriorated and the reactivity of its vessels diminished

Even if there exists a certain disagreement regarding the extent of the resistance changes in the intestine upon hemorrhage there is general agreement that the flow resistance in the extremities is markedly increased upon a blood loss (see e g ECKSTEIN LIEBOW and WIGGERS 1946 GREGG 1962 ABEL and MURPHY 1962) In most earlier investigations of flow resistance changes in limbs a fairly big bulk of skin tissue has been included in the preparation studied Considering the fact that the cutaneous vessels evidently do not participate to any substantial extent in the reflex adjustments to hemorrhage it seems justified to conclude that the reflex changes in skeletal muscle flow resistance must have been of a substantial magnitude in these studies A recent study on the effects of severe hemorrhage upon an isolated skeletal muscle vascular circuit (POTHE SCHWENDENMANN and SELKURT 1963) seems to support such a view

A constriction of the capacitance vessels and an expulsion of blood from the various organs was found regularly in this study when a sudden blood loss was induced This is in keeping with the studies of ALEXANDER (1955) in which hemorrhage was associated with a reflex increase of venous tone in an intestinal vascular bed PEYNELL *et al* (1956) JOHNSON and SELKURT (1958) and FRIEDMAN (1961) likewise found that splanchnic and intestinal blood volumes decreased markedly in hemorrhage evidently due to an expulsion of blood from the capacitance vessels On the other hand no clear evidence of an enhanced venous tone have been obtained in human beings upon pooling of blood in peripheral vascular sections (see e g GAUER and THRON 1962 GAUER and HENRY 1963) The reason for this might be that these studies were performed mainly on tissue parts where the greater fraction of the blood content can be expected to be contained in the cutaneous vascular bed (hand and foot) The cutaneous vessels as mentioned earlier seem to be relatively little involved in reflex cardiovascular mechanisms but are presumably primarily engaged in the regulation of body temperature This vascular bed is therefore probably not representative of other vascular circuits as those in skeletal muscles and intestine

The present experiments also indicate that the reflex mechanisms responsible for the resetting of the pre to postcapillary resistance ratio and the conse

quent transcapillary fluid exchange possess a high degree of sensitivity. Clearcut reflex adjustments were observed especially in the skeletal muscles when only 2—3 per cent of the total blood volume was shed. Therefore the cardiovascular receptors involved in these compensatory reflex mechanisms seem to be capable of "detecting" even minute changes in wall distension in those parts of the cardiovascular system where they are situated. When all receptors were intact these vascular responses could be obtained even if mean arterial blood pressure was not changed to a discernible extent. This does not, of course, exclude the possibility that the arterial baroreceptors were involved in the production of the abovementioned compensatory vascular adjustments, as a change in pulse amplitude consequent upon, e.g., a reflex change of heart rate will considerably influence the activity of the baroreceptors even if mean arterial pressure is not changed (EAD, GREEN and NEIL 1952).

According to GAUER and HENRY (1963) a minor blood loss is likely to influence primarily the receptors located in the heart and adjacent parts of the low pressure system. Because of the pronounced distensibility of this vascular section, even minute pressure changes might alter the distortion of their walls and hence affect the receptor activity. Those changes in central venous pressure might in fact be so small that they would hardly at all affect the capillary pressure level in a retrograde direction and hence not be able in themselves to affect the fluid transfer across the capillaries in such a way.

On the other hand, the present findings that a denervation of the heart receptors did not reduce but often rather enhanced the reflex vascular adjustments may at least at first sight appear as a contradiction. However, according to preliminary experiments presented elsewhere (ÖBERG 1963 *a*) it seems that the receptors located in the heart and adjacent vascular areas reflexly affect the vascular bed to a relatively smaller extent than the heart, to judge from their fairly extensive influence on heart rate as contrasted to their relatively small effect on blood vessels. In these mentioned experiments, performed on anesthetized, curarized and artificially ventilated cats, atropine was given initially so that the sympathetic influence on the heart was the only intact efferent pathway for inducing reflex shifts in heart rate. Direct stimulation of the vagal nerves in the efferent direction at the end of the experiment revealed no change in heart rate, proving that the vagal block was complete. Sectioning the vagal nerves therefore will not imply a change of the efferent innervation of the heart. Standardized hemorrhages were then produced before and after elimination of either the intrathoracic receptor areas, by cutting or cooling the vagal nerves including the aortic depressor nerves, or the carotid receptors, by cutting the sinus nerves. It was found

that a graded blood loss produced rather weak increases in heart rate but relatively pronounced increases of flow resistance when the afferent vagal fibres but not the sinus nerves had been blocked while the reverse was true if the sinus nerves were blocked selectively The extent of the blood pressure fall was of comparable magnitude in the two cases These findings were confirmed *in the course of the present experiments and they suggest that the* intrathoracic cardiovascular receptors reflexly affect the sympathetic discharge to the heart to a relatively greater extent than do the receptors in the carotid artery while their reflex effects upon the vessels are relatively less pronounced

Such a more dominant influence of the intrathoracic receptors on the heart as compared with carotid sinus baroreceptors was suggested by WINDER (1937) although he considered the aortic baroreceptors rather than the cardiac receptors to be of prime importance in this respect However the reflex response pattern observed upon injection of veratrum alkaloids which in the concentrations used appears to excite only *the heart receptors* points to the heart receptors as being probably the main origin for these relatively more marked reflex effects upon heart rate (see Chapter III)

If such a relatively preferential reflex engagement of the heart is indeed elicited from the heart receptors this would imply that minute blood losses assumed to affect primarily these particular receptors (GAUER and HENRY 1963) would produce an increased heart rate and a mobilization of the blood depot available in the heart itself by way of reflex modulations of the activity in its vagal and sympathetic fibres It is likely that a complex interaction between heart receptors and arterial receptors occurs which calls for some consideration It is possible *for instance that after a small blood loss* the heart receptors by their preferential reflex effects on the heart itself might so effectively maintain a nearly normal cardiac output arterial pressure and even pulse amplitude that the arterial receptors would be only insignificantly affected Only minute vascular adjustments would then occur However if the same amount of blood loss is induced when the heart receptors are denervated it may be expected that the reflex activation of the heart would be less extensive Then cardiac output and arterial pressure would tend to fall more significantly and *via* the arterial receptors more considerable vascular adjustments would ensue Such an interaction between the two types of receptor *sites might best explain the present at first sight puzzling* observation that the reflex vascular adjustments were often intensified after denervation of the intrathoracic cardiovascular receptors while then the reflex increase of heart rate was less pronounced It might also explain why sometimes when all receptors were intact a blood loss produced reflex vas

The principal factors determining the rate and direction of the net fluid transfer across the capillary membranes and the possible means by which this process can be reflexly influenced were discussed in *Chapter I* A brief survey of the pertinent literature was given the aim of the present investigation was defined and the principles of the experimental approach outlined briefly

The methods and experimental procedures utilized in this study were described and critically discus ed in *Chapter II* The net transcapillary fluid exchange in various tissues was followed continuously by means of a plethysmographic registration of changes in tissue volume The characteristic slow but steady volume change occurring when no apparent shifts in vascular tone were going on was used as an indicator of the direction and rate of the net transcapillary fluid exchange On the other hand rapid phasic shifts in tissue volume occurring at the same time as the changes in flow resistance were considered to reflect changes in regional blood content consequent upon alterations in vascular tone These occur primarily within the venous compartment since it constitutes the most important part of the capacitance section — The blood flow through the tissue studied was measured continuously from the venous effluent Arterial inflow and venous outflow pressures in the vascular beds studied could be adjusted at will and set at any desired level This permitted a direct evaluation of the reflexly induced adjustments in the pre to postcapillary resistance ratio from the changes in direction and in rate of the net transcapillary fluid exchange The capillary filtration coefficient (CFC) reflecting the size of capillary surface area available for the filtration and absorption processes could be determined repeatedly and was then considered to provide information concerning shifts in the tone of the precapillary sphincters — From these recorded parameters it was then possible to follow continuously and to a certain extent quantitatively the reactions of the resistance capacitance and precapillary sphincter sections of a vascular circuit as well as the shifts in the ratio between the pre and postcapillary resistances — The different types of cardiovascular reflex mechanisms studied and the methods used for receptor stimulation and inactivation were also described in this chapter

In *Chapter III* various individual cardiovascular reflex patterns were studied with special interest focussed upon induced reflex shifts in the net fluid exchange across the capillary membranes and in *Chapter IV* the effects of hemorrhage were similarly analysed It was found that a reflex activation of the vasoconstrictor fibres due to baroreceptor inhibition chemoreceptor stimulation or hemorrhage induced a significant absorption of fluid into the blood stream in skeletal muscles and in the skin concomitantly with a reflex

constriction of the resistance and capacitance vessels in all the tissues studied The rate of the fluid absorption was correlated with the extent of reflex vasoconstrictor fibre activation as judged from the changes in flow resistance Thus the more the intrasinusal pressure was reduced or the larger the hemorrhage was the faster was the fluid absorption Conversely a reflex inhibition of the vasoconstrictor fibre activity following e g a stimulation of the baroreceptors or the heart receptors induced a net filtration of fluid from the blood into the interstitial spaces in skeletal muscle and skin associated with a dilatation of their resistance and capacitance vessels The rate of net filtration was also well correlated to the degree of the reflex vasoconstrictor fibre inhibition The recorded movements of fluid must under the prevailing experimental conditions be ascribed to changes in mean capillary pressure consequent upon alterations in the pre to postcapillary resistance ratio Reflex alterations of the vasoconstrictor fibre discharge to the vascular beds in skeletal muscle and skin will thus regularly induce more pronounced changes of flow resistance in the precapillary vascular section than in the postcapillary one both absolutely and relatively This results in well graded shifts in the pre to postcapillary resistance ratio and thereby in the capillary pressure in these tissues

In the intestinal vascular bed on the other hand no net transcapillary fluid exchange was ever initiated by reflex shifts in vasomotor fibre activity as long as the intestinal preparation was in a good condition with well maintained vascular reactivity These findings indicate that in this tissue the pre to postcapillary resistance ratio and hence the capillary pressure remained essentially constant with reflex shifts in the vasoconstrictor fibre activity The reason for this was considered to be the marked tendency of the intestinal precapillary vessels to undergo secondary locally induced adjustments which have been called autoregulatory escape from the constrictor fibre influence Therefore, despite continued enhanced vasoconstrictor fibre activity the intestinal precapillary resistance was in the steady state phase of the reflex vascular adjustment increased only to an extent which exactly balanced the relatively small postcapillary resistance increase This phenomenon and its probable background was discussed in more detail in Chapter III

It was a general observation in the present experiments that the resistance vessels in the different parallel coupled vascular circuits were not affected to the same extent by baroreceptor reflex mechanisms or after hemorrhage The resistance vessels in the skeletal muscles were found to be more strongly affected than those of the intestine or the skin This is accordance with earlier observations by LOFVING (1961 *a b*) As discussed in Chapter III the background for this quantitative differentiation is probably not the same in all

that the relative role of intrathoracic receptors is more dominant in man considering the fact that his upright position might have called for quantitative changes in the overall organization of reflex cardiovascular control (see e g RODDIE and SHEPHARD 1958)

In any case a more detailed analysis of reflex vascular adjustments has thus revealed that an appropriate control of the functionally different consecutive sections in a vascular circuit can contribute considerably to the maintenance of circulatory homeostasis in various ways when the normal balance between vascular holding capacity and blood volume is upset Reflex shifts in the tone of the resistance vessels in the various parallel coupled circuits will then adjust the distribution of the cardiac output so as to favour the most vital organs e g the brain and myocardium (cf HEYMANS and NEIL 1958 FOLKOW 1960) Reflex alterations in the tone of the capacitance vessels will influence total vascular capacity and hence influence directly venous return to the heart Finally, reflex resetting of the pre to postcapillary resistances in appropriate tissues mainly skeletal muscles and to some extent skin will change the net fluid exchange across the capillary membranes so that the plasma volume can be increased or decreased at the expense of the fairly extensive interstitial fluid reservoirs of these tissues These different compensatory reactions require however variable time lapses to display their full effects Adjustments of vascular capacity and flow resistance can be accomplished *immediately* after e g a blood loss because of the promptness of the reflex influences on vascular smooth muscle tone and will then constitute a sort of first line of defence A mobilization of extravascular fluid is simultaneously initiated from the skeletal muscle and from the skin by means of reflex resettings of the pre to postcapillary resistance ratio However since the ensuing fluid absorption is relatively slow a certain time must pass before the plasma volume is significantly affected This compensatory mechanism can therefore be regarded as a second line of defence since its consequences will appear only more gradually but with time after a blood loss it will obviously be of increasing importance A still more slowly acting third line of defence consists of the compensatory restriction of the continuous fluid and salt losses from the organism *via* the kidneys by means of a reflex augmentation of the release of the antidiuretic hormone (see e g GAUER and HENRY 1963) Even if this latter mechanism is of tremendous importance for the *long term* regulation of the total fluid content in the organism it cannot by itself substantially contribute to a rapid restoration of plasma volume upon sudden blood losses since no real *gain* of circulating fluid volume can be accomplished Whether the volume receptors which are assumed to affect the kidney function are also responsible for the thirst following a blood

loss and in this way aid in fluid restoration is not known (GREGERSEN and RAWSON 1959)

The functional significance of the observation that a substantial net fluid exchange could be obtained especially in the skeletal muscles and to some extent in the skin in connection with the cardiovascular reflexes studied while no such fluid shift occurred in the intestine is difficult to evaluate in all its aspects and such an evaluation will of necessity involve some speculations It is however obvious that the skeletal muscles are especially well suited to serve as an extravascular fluid reservoir Because of their large total mass they contain a large volume of interstitial fluid even if the relative amount calculated per unit weight of tissue seems to be smaller than in many other tissues (see e g HARRISON DARROW and YANNET 1936) Therefore even a relatively moderate mobilization of the interstitial fluid content from the skeletal muscles consequent to a reflex reduction of mean capillary pressure will result in a substantial gain in the intravascular fluid content This may be visualized with an example based on actual figures from the present experiments extrapolated to man A human being weighing 70 kg may in thirty minutes increase his plasma volume by roughly 300—400 ml merely by means of a reflexly induced fluid absorption from the skeletal muscle extravascular space if the capillary pressure is reflexly lowered 4—5 mm Hg and CFC is assumed to be on an average 0 01 ml/min/100 g/mm Hg in this tissue A reflex lowering of the capillary pressure of this magnitude in the skeletal muscles is by no means unlikely after a blood loss and implies an increase of flow resistance in this tissue of approximately 100 per cent according to the present experiments In terms of the resulting decrease of fluid content in the interstitial space in the muscle mass it would mean that about 10 per cent of this fluid store needs to be transferred to the intravascular compartment to accomplish this fairly large autotransfusion Shifts in interstitial fluid volume of this magnitude can evidently occur in skeletal muscles without any significant concomitant changes in tissue pressure (KJELLMER 1964) Furthermore a disturbance of the normal transcapillary pressure equilibrium necessary for a net fluid exchange between blood and tissue spaces can be readily induced and well maintained in the skeletal muscles Even the marked and sustained vasoconstriction required to produce more powerful and lasting shifts in the pre to postcapillary resistance ratio and hence in capillary pressure will not interfere significantly with the local demands of this tissue as resting skeletal muscles evidently tolerate relatively wide variations in their blood supply Similar favorable conditions for extravascular fluid mobilization are presumably also present in the skin though it should be realized that the demands of temperature control may often

overpower the receptor induced vascular adjustments in this tissue It should however be noted that this fluid mobilization as studied in the present experiments has its limitations and that the fluid absorption must be considered as a self limiting process The continuous dilution of plasma proteins and the gradual increase of tissue colloid osmotic pressure will thus tend to lower the colloid osmotic pressure gradient across the capillary walls and hence progressively reduce the rate of fluid absorption Moreover when the vascular compartment becomes refilled the extent of the reflex adjustments responsible for the absorption can be expected to decline

The *intestine on the other hand is a small tissue compared with the skeletal* muscles and contains therefore only a fairly small fraction of the total body interstitial fluid Even if CFC in the intestine is some 10 times greater than in the skeletal muscles so that even small shifts in capillary hydrostatic pressure will produce considerable fluid transfers across the capillary membranes the absolute volume of fluid that might be mobilized from the gastro intestinal tract would therefore in relation to the total plasma volume be fairly insignificant On the other hand if the capillary pressure should *increase* in the intestine in connection with a reflex inhibition of sympathetic activity, the high filtration coefficient of this tissue would constitute a considerable potential hazard for massive edema formation if no other mechanisms were present to protect against such an event The autoregulatory adjustments occurring in the precapillary resistance section and by which the capillary pressure in the intestine is normally kept surprisingly constant during various vascular adjustments might therefore be looked upon as a local safeguard *against such hazards It was in fact occasionally observed that this* auto regulatory mechanism ultimately failed along with a deterioration of the intestinal preparation and then edema formation occurred rapidly in the intestine with reflex inhibition of the vasoconstrictor fibre activity However absorption of fluid from the extravascular space of the intestine can occur if the portal and the central venous pressures and hence the intestinal capillary pressure are lowered in connection with the blood loss but owing to the small tissue mass only a relatively small amount of fluid can be expected to be mobilized from the intestine

The significance of the earlier findings (*e g* LOFVING 1961 *a b*) that reflex increases in flow resistance are most readily induced in the skeletal muscles and generally more pronounced in this tissue than in intestine skin or kidney might be more easily understood in the light of the present experiments If one considers the reflex reactions of the resistance vessels in individual circuits as a means of regulating only the total flow resistance *per se* it would appear more reasonable to have a *generalized* constriction in those

circuits which normally receive a considerable fraction of the cardiac output so as to favour the blood supply to the brain and the myocardium Admittedly such more generalized resistance responses might be seen when the vasomotor centre excitation is very intense as in emergency situations following a gross hemorrhage a severe asphyxia etc However at more moderate sympathetic discharge rates as occur with small blood losses local pooling of blood etc significant reflex adjustments of the resistance vessels seem to take place practically only in the skeletal muscles at least in the cat (cf FOLKOW JOHANSSON and LOFVING 1961) The shifts in *total* flow resistance produced by such a relatively selective engagement of the skeletal muscle vessels may in fact be expected to be quite small For instance if their flow resistance should be doubled and no simultaneous significant engagement of the other circuits occurred the overall flow resistance should increase by only 10 per cent since no more than approximately 20 per cent of the total cardiac output goes to the skeletal muscles during rest Therefore the relative differentiation of the reflex control of the various systemic circuits with a preference for skeletal muscle vessels seems to be of little advantage if the primary aim were to adjust the total flow resistance in order of favour the blood supply to the brain and the myocardium This extent of reflex adjustment in the skeletal muscle vessels can on the other hand be expected to produce a substantial mobilization of fluid into the circulation because of the reflex resetting of the pre to postcapillary resistance ratio in this big tissue mass It would therefore appear more reasonable to assume that the mentioned differentiated reflex control of the muscle resistance vessels is not designed primarily for altering overall flow resistance but rather to establish possibilities for a reflex control of fluid volume in the circulatory system by affecting the net exchange of fluid between the blood and the extravascular compartment in this tissue This would imply a functional organization which makes it possible for the baroreceptors to exert an effective and fairly specific control of the fluid partition between the extra and intravascular compartments and hence of the blood volume That some sort of a true control must exist here seems clear from the well recognized fact that the blood volume and then evidently the distribution of the extracellular fluid is maintained so surprisingly constant from moment to moment despite a multitude of disturbing factors The capillary pressure will thus inevitably be subjected to substantial variations in daily life due to hydrostatic factors upon shifts in body position etc with consequent net movements of fluid between blood and tissue spaces Whenever the intravascular content changes significantly compensatory reflex shifts in the fluid exchange in the skeletal muscles can be induced readily and this without

any substantial interference with overall cardiovascular dynamics or with the blood supply to other tissues

The present finding that a net filtration of fluid from the blood to the skeletal muscles occurred upon reflex inhibition of the prevailing vasoconstrictor activity or after an acute denervation of the vessels might seem contradictory to earlier observations that the blood volume in fact *increases* in sympathectomized animals (HAMLIN and GREGERSEN 1939 CHEN 1958) since the pre to postcapillary resistance ratio ought to increase upon this procedure at least in muscle and skin However when the entire sympathetic nervous system is blocked, arterial and venous pressures generally fall considerably which *per se* tend to reduce the capillary pressure Whenever this type of influence on capillary pressure overbalances the oppositely directed influence of the reduced pre to postcapillary resistance ratio the capillary pressure will on the average be reduced leading to a fluid absorption and hemodilution Further fairly soon after sympathectomy the precapillary resistance vessels appear to regain their tone (*see e g* BARCROFT and SWAN 1953 p 58) which in this situation will then aid in keeping mean capillary pressure reduced On closer view it is therefore quite reasonable that a certain reabsorption of tissue fluid should occur after a sympathectomy, helping to increase the blood volume It should of course also be realized that the blood volume is influenced by several mechanisms and is therefore in the long run not necessarily related only to the rate and direction of fluid transfer across the capillaries in muscle and skin

On the whole the fact that mean capillary pressure might be reduced after sympathectomy by no means invalidates the present finding that an effective reflexly governed control of the filtration exchange is exercised by means of appropriate modulations of the pre to postcapillary resistance ratio in skeletal muscles and skin, where an inhibition of sympathetic activity causes a filtration loss from the intravascular compartment and *vice versa* It should be remembered that in the intact organism a reflex decrease of this ratio tending to raise capillary pressure and produce filtration occurs when arterial and/or venous pressures tend to be raised due to an overfilling of the system and when therefore a fluid loss from the circulation might be of benefit Under these circumstances the raised venous pressure cooperates with the reflex decrease of the pre to postcapillary resistance ratio in causing a net filtration transfer The reverse situation seen *e g* with blood loss where capillary pressure is reflexly lowered and reabsorption ensues is also characterized by such a cooperation between these two mechanisms in lowering the capillary pressure The situation met with in the intact organism exposed to blood loss or overfilling of the cardiovascular system is therefore entirely different from that seen on acute block of the sympathetic nervous system where the en

suing fall in arterial and central venous pressures *opposes* the pre- to post-capillary ratio decrease in terms of its effect on mean capillary pressure.

However, even if reflex adjustments of the fluid partition between the extra- and intravascular compartments in the body can contribute to a considerable extent to the maintenance of a proper relation between vascular holding capacity and content, their delayed action makes them of secondary importance in the very *acute* phase of, *e.g.*, a sudden blood loss. Long before an effective autotransfusion of tissue fluid can be produced a cardiovascular collapse may have ensued if the blood loss is sudden and profound. A reflex mobilization of the blood depots in the capacitance vessels and in the heart, which can occur instantaneously, is evidently a more important mechanism in the acute situation.

It might on first sight appear that the most appropriate control device serving to maintain the cardiac output and blood supply to the various tissues in connection with at least minor blood losses should be a *selective* reflex control of the discharge to the heart and the capacitance vessels without any significant shift in fibre activity to the resistance vessels (NEIL 1962 *a*). A tightening of the cardiovascular system around its blood content could then be brought about without any significant blood flow restrictions to the different tissues. However, if a *selective* activation of the constrictor fibres to the venous section occurred, it would seem inevitable that the pre- to postcapillary resistance ratio would, if anything, be decreased, tending to produce a slow fluid loss into the tissues. In the long term this would tend to counteract the beneficial effect of a venous constriction aimed at combatting a fluid loss. Moreover, in such a situation no device for correcting fluid loss would be present and the cardiovascular system would operate in a state of temporary compensation only by utilizing the reserves inherent in intravascular depot mobilization.

On the other hand, it should be realized that a substantial reflex constriction of the capacitance vessels might be brought about with only *minor* concomitant increases in the postcapillary flow resistance. Therefore only fairly small shifts in precapillary (and hence in total) flow resistance would be required to overbalance the effects of venous constriction on the capillary pressure and produce a net fluid absorption. In fact, the frequency-response curves for the heart (FOLKOW, LOFVING and MELLADER 1956; CARLSTEN, FOLKOW and HAMBERGER 1957) and for the resistance and the capacitance vessels (MELLANDER 1960) have such slope characteristics that even a *uniform* change in fibre discharge in the low frequency range will produce the relatively more dominating effects on the heart and the capacitance vessels with fairly small concomitant effects on total flow resistance. Nevertheless, in skeletal muscle and skin the precapillary resistance is then increased enough

to overbalance the slight postcapillary resistance increase which always must ensue upon a venous constriction (MELLANDER 1960) and in the intestine an exact balance between pre and postcapillary resistances occurs (FOLKOW *et al* 1964 *a*)

Even if a disturbance of the normal relation between vascular fluid content and capacity is seen in its most drastic form in an acute blood loss it should be realized that in principle the same situation will be created in a number of physiological situations as during profuse sweating gastrointestinal secretion etc leading to temporary fluid losses from the intravascular compartment which have to be replaced from extravascular sources The upright posture in humans will lead to a considerable pooling of blood in dependent vascular beds and a diminution of the effective circulating blood volume Furthermore due to the massive hydrostatic load imposed upon the capillaries from both the venous and the arterial sides in dependente body parts the capillary pressure will in this situation rise markedly creating possibilities for an extensive filtration of fluid from the circulation which would further diminish venous return Appropriate compensatory mechanisms directed towards diminishing both the pooling of blood and the fluid losses across the capillary wall are thus badly needed in this situation Reflexly induced changes of venous tone will stiffen the venous walls and counteract too marked accumulations of blood in dependent regions and will also help to mobilize blood from other vascular circuits A reflex increase in the pre to postcapillary resistance ratio will tend to reduce the rate of outward filtration in dependent tissues and at the same time induce a fluid absorption in other vascular beds which are not exposed to a high hydrostatic pressure However even a very marked rise of the pre to postcapillary resistance ratio can limit the rise of capillary pressure in the dependent regions to only a small extent as the pressure is raised on the venous side too On the other hand a constriction of the precapillary sphincters can in this situation drastically reduce the rate of fluid loss from the circulation by restricting the capillary surface area available for the filtration into the extravascular compartment (MELLANDER ÖBERG and ODELRAM 1964) This constriction of the precapillary sphincters seems to be basically independent of reflex influences and is probably caused by a reinforcement of the rhythmic myogenic activity of the sphincters following the increase in transmural pressure (see *e g* FOLKOW 1962) This locally induced adjustment of the capillary surface area in dependent vascular beds which will be automatically well adjusted to the regional rise in transmural pressure may be considered as one of the most important mechanisms by which the fluid losses in dependent body parts can be restricted when the body assumes the upright position

Summary

The effects of various cardiovascular reflexes upon the net fluid exchange across the capillary membranes in skeletal muscle skin and intestine have been studied in chloralose anesthetized cats The fluid movements between the intra and extravascular compartments were followed by continuous measurements of tissue volume changes with a plethysmographic technique This method combined with measurements of blood flow and arterial and venous pressures also allowed for a study of the reflex adjustments of the resistance vessels the capacitance vessels and the precapillary sphincters

A reflexly induced augmentation of the vasoconstrictor fibre activity (produced by inactivation of the arterial baroreceptors stimulation of the chemoreceptors or hemorrhage) was consistently found to induce a net *absorption* of tissue fluid into the circulation from *skeletal muscle and skin* The rate of this absorption was closely related to the extent of the concomitantly occurring constrictions of the resistance and capacitance vessels Conversely a reflex inhibition of vasoconstrictor fibre activity (produced by stimulation of the arterial baroreceptors or the heart receptors) induced a dilatation of the resistance and capacitance vessels in the tissues studied and regularly a net *filtration* of fluid into the interstitial space in *skeletal muscle* and *skin* In contrast *no* discernible net fluid transfer occurred across the capillary membranes in the intenstine concomitant with reflex adjustments of the resistance and capacitance vessels

The observed movements of fluid between intra and extravascular compartments must be ascribed to alterations in mean capillary pressure consequent to reflex resettings of the ratio between the flow resistances in the precapillary and postcapillary vascular sections respectively Thus a reflex enhancement of the vasoconstrictor fibre activity lowered the capillary pressure in skeletal muscle and skin by increasing the pre to postcapillary resistance ratio This implies that a more marked increase in precapillary than in postcapillary resistance occurred both in absolute and relative terms Conversely a reflex inhibition of the prevailing vasoconstrictor fibre activity was followed by a rise of the capillary pressure in skeletal muscles and skin due to a relatively more marked reduction of the precapillary than of the postcapillary resistance

In the intestine on the other hand there was a remarkable constancy of the capillary pressure in the reflex vascular adjustments investigated This

seems to be due to potent 'autoregulatory' adjustments which modify the neurogenically induced shifts in tone in the intestinal precapillary vessels in such a way and to such an extent as to keep the pre to postcapillary resistance ratio constant over a wide range of changes in constrictor fibre activity

Quantitative differences were observed with regard to the reflexly induced vascular adjustments in the various tissues studied The reflex changes in total flow resistance and pre to postcapillary resistance ratio were throughout most pronounced in the skeletal muscles leading to especially marked reflex shifts in mean capillary pressure and net transcapillary fluid exchange in this tissue The net absorption of fluid in the skeletal muscles occurring when the vasoconstrictor fibres were reflexly activated was further enhanced by a concomitant relaxation of the precapillary sphincters leading to an enlargement of the capillary surface area available for the fluid exchange process This dilatation of the precapillary sphincters was in all probability due to the action of vasodilator metabolites accumulated in the tissue during the reflex reduction of blood flow

Due to the large tissue mass of the skeletal muscles and the marked and well sustained reflex vascular adjustments in this tissue considerable volumes of fluid can be transferred from their extravascular space into the circulation (and vice versa) with only small shifts in the vasoconstrictor fibre discharge

The receptor governed influence on the pre to postcapillary resistance ratio in the skeletal muscles and to some extent also in the skin thus constitutes a potent and sensitive device for a control of the fluid partition between the intra and extravascular compartments tending to keep the volume of circulating fluid constant By means of this specific reflex adjustment a fall in capillary pressure and a fluid replacement after a blood loss can be accomplished even before any drastic changes in arterial and central venous pressures have occurred

The cardiovascular receptors engaged in this type of control of plasma volume seem to be very sensitive to even small reduction in the volume of circulating fluid A blood loss corresponding to only a few per cent of the total blood volume was thus often found to be sufficient to reflexly initiate a fluid absorption in the skeletal muscles It seems as if all the cardiovascular receptors studied are engaged in this compensatory response to a blood loss but some data indicate that at least in the cat the arterial receptors are of relatively greater importance than the heart receptors for inducing vascular adjustments including a resetting of the pre to postcapillary resistance ratio in appropriate tissues The heart receptors seem to be of special importance for producing reflex shifts in heart activity though they no doubt also contribute to the vascular adjustments

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AEROBIC WORK CAPACITY AND CIRCULATION AT EXERCISE IN MAN

With special reference to the effect of prolonged exercise and/or heat exposure

By

BENGT SALTIN

STOCKHOLM 1964

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Translated
by
Joan Blomqvist

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The present publication is based on studies reported in the following papers

I Oxygen uptake during the first minutes of heavy muscular work Åstrand, P O and B Saltin *J appl Physiol* 1961, *16* 971—976

II Maximal oxygen uptake and heart rate in various types of muscular activity Åstrand, P O and B Saltin *J appl Physiol* 1961, *16* 977—980

III Cardiac output during submaximal and maximal work Åstrand P O T E Cuddy, B Saltin and J Stenberg *J appl Physiol* 1964 *19* 268—274

IV Circulatory response to submaximal and maximal exercise after thermal dehydration (with a comparison between the acetylene and the dye dilution method for determination of cardiac output) Saltin, B *J appl Physiol* 1964, *19* In press

V Plasma and red cell volume after prolonged severe exercise Åstrand P O and B Saltin *J appl Physiol* 1964, *19* In press

VI Effect of sweat loss on body fluids Kozlowski S and B Saltin *J appl Physiol* 1964, *19* In press

VII Blood lactates after prolonged severe exercise Åstrand P O I Hallback R Hedman and B Saltin *J appl Physiol* 1963 *18* 619—622

VIII Aerobic and anaerobic work capacity after dehydration Saltin B *J appl Physiol* 1964 *19* In press

IX Circulatory response to prolonged severe exercise Saltin B and J Stenberg *J appl Physiol* 1965, *20* In press

In the text these papers will be referred to by Roman figures I—IX The figures and tabels in the present publication are either taken directly from or based on data first published in above mentioned papers

PREFACE

This study was carried out during the years 1959—1964 and is to a great extent the result of team work. My present superior, Docent PER OLOF ÅSTRAND, has been a source of inspiration and every phase of the study has developed and been brought to completion through his stimulating guidance. I wish to express my sincere thanks for his advice and never faltering active help. I am also greatly indebted to my former superior Professor E. HOHWÜ CHRISTENSEN, who introduced me to physiological research and who, despite being abroad during the work's execution, followed the study and gave worth while advice.

I would also like to express my warmest thanks for the invaluable help received throughout this study from the personnel at the Department of Physiology at GCI.

The subjects' cooperation and motivation in performing the extremely trying tests was an essential factor for the completion and worth of this work. For this I am deeply grateful to them.

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Stockholm 1964 BENGT SALTIN

INTRODUCTION

Several interdependent factors are important for a good psysical work capacity In general the following points are essential

Energy output	*aerobic processes* anaerobic processes
Neuro muscular function	strength technique
Psychological factors	

In many situations such as after prolonged work and after dehydration physical work capacity is decreased The question arises which of the above factors has been effected *Aerobic processes play a dominant role in energy* production during prolonged heavy muscular work In the present paper therefore the emphasis is placed upon investigation of the oxygen transporting organs particularly upon the circulation at various levels of oxygen uptake

The study began with an investigation of how work time and type of muscular work influence the measured maximal oxygen uptake (I II) An analysis of the central circulation during heavy muscular work is included also in the METHOD section (III IV) The next question then is how extended heavy muscular work and heat exposure with resulting dehydra*tion in both cases, influence the body s various fluid compartments (IV V* VI IX) as well as the central circulation and the aerobic work capacity (IV VII VIII IX)

In sections V and VII results are presented based on field studies after ski competitions The field studies gave worthwhile experimental conditions impossible to duplicate in the laboratory

The subsequent exposition is divided into Part I (I, II III IV) and Part II (IV V, VI VII, VIII IX) Part I mainly concerns methodological questions Each section is introduced by a short background sketch The intention here was to clarify some of the factors essential to a comprehension of this series of papers References are given mainly to reviews in which more detailed bibliographies are available

PART I

BACKGROUND

Determination of Maximal Oxygen Uptake

The determination of maximal oxygen uptake has been of interest to investigators for the past 50 years (Benedict and Cathcart 1913) In the classical work dating from the 1920 s it was established that there exists an interindividual variation in maximal oxygen uptake (Hill and Lupton 1923) which is crucial for the ability to perform prolonged muscular work (Hill Long and Lupton 1924 Bock Vancaulaert Dill Folling and Hurxthal 1928 b Herbst 1928 P O Åstrand 1956) Herbst (1928) demonstrated how oxygen uptake upon a gradual increase in running speed attained a maximal level, any subsequent increase in tempo induced no further rise in oxygen uptake (apparent steady state) This levelling off has been used as the criterion for the attainment of a maximal value (P-O Åstrand 1952 ch 3, Taylor Buskirk and Henschel 1955) In cases in which a levelling off was not observed, accumulation of lactic acid in the blood has been of help in evaluation (P-O Åstrand 1952 ch 3 8 I Åstrand 1960 Part I) Both these criteria for the determination of the maximal level have been critized recently by Wyndham Strydom Maritz Morrison Peter and Potgieter (1959), they are of the opinion that the error of the determination of the maximal oxygen uptake can be very great when using the present criteria To decrease this error it is necessary, according to Wyndham et al to determine the relationship of work to oxygen uptake on several separate occasions (the method actually used by us Åstrand 1952 For a critical discussion of Wyndham et al s work see I Åstrand 1960 (pp 59—60)

Hill and Lupton (1923) concluded that of all the oxygen uptake determinations done during hard work up to then the highest values were obtained in running skiing and skating Large enough muscle groups are utilized during these activities to load the oxygen transport system to the maximum The critical size of these large muscle groups has not been specified Maximal arm work yields a lower oxygen uptake than does leg work (Christensen 1932 a Asmussen and Hemmingsen 1958) Upon comparison of maximal bicycling with running P-O Åstrand (1952 ch 9) found no difference in oxygen uptake Taylor et al (1955) had a subject perform arm work while running Oxygen uptake increased by 0 2 l/min or 7 % This was in agreement with Åstrand (1952 p 119) and Christensen and Hogberg (1950) who on a subject in common found a higher oxygen uptake in maximal skiing than in running and bicycling

Cardiac Output During Work

Cardiac output occupies a central position in the discussion of oxygen transport from the lungs to the tissues The size of the cardiac output at rest and during work has been open to speculation and investigation ever since Harvey (1628) developed his argument for a closed circulation within the body s blood vessels

Not until the first decades of the 20th Century were the technical difficulties mastered in measuring human cardic output (Loewy and Schrotter 1905) The first mapping of cardiac output during work was done by KROGH and LINDHARD (1912) and LINDHARD (1915) Various methods according to BORNSTEIN s principle (1910) or indirect Fick as well as the use of various inert gases (N O C Hc and C H) gave partially differing results which initiated criticism and doubt as to the reliability of the different methods of determination (LINDHARD 1915, BOOTHBY 1915 DOUGLAS and HALDANE 1922 HENDERSON 1925 BOCK DILL and TALBOTT 1928 a BOCK et al 1928 b MARSHALL and GROLLMAN 1928 GROLLMAN 1929 CHRISTENSEN 1931 a 1932 b GROLLMAN PROGER and DENNING 1931 CHRISTENSEN and TROLLE 1933)

With the determination of cardiac output by the dye dilution method or direct Fick however a more conclusive evaluation of earlier determinations has been arrived at thereby reinstating the foreign gas method (ASMUSSEN and NIELSEN 1952 WADE and BISHOP 1962 ch 3 compare however WERKO BERSEUS and LAGERLOF 1949 CHAPMAN TAYLOR, BORDEN EBERT KEYS and CARLSON 1950)

The circulatory parameters responsible for oxygen transport (from the lungs to the body tissues) can be expressed algebraically according to Fick s equation

oxygen uptake	= heart rate	× stroke volume	× arterio venous oxygen difference
ml/min	beats/min	ml/beat	vol %

The relative importance of the involved parameters on a gradual increase of oxygen uptake has been comparatively well studied in light to moderate exercise Literature is beginning to accumulate for heavy work but it is not yet possible to draw any definite conclusions A large number of studies has demonstrated a linear relationship between heart rate and oxygen uptake up to the maximal level (for example CHRISTENSEN 1931 b P O ÅSTRAND 1952 ch 11 MITCHELL SPROULE and CHAPMAN 1958 compare however with Wyndham et al 1959) Whether or not there is an increase during work in the stroke volume contributing to the increase in the cardiac output has not been settled (see RUSHMER and SMITH 1959 WADE and BISHOP 1962 ch 3 BEVEGÅRD 1962) The rise in stroke volume is in-

consequential during work in a supine position (WANG, MARSHALL and SHEPHERD 1960, HOLMGREN, JONSSON, SJOSTRAND 1960, BEVEGÅRD, HOLMGREN, JONSSON 1960 and 1963, GRIMBY and NILSSON 1963). MUSSHOF, REINDELL, STEIM and KONIG (1959) found, however, a certain increase (max 60 %) during light work (Vo < 2.0 l/min). In upright work position the stroke volume increases markedly upon transfer from rest to work (CHRISTENSEN 1932, MITCHELL et el 1958, WANG et al 1960, CHAPMAN, FISCHER and SPROULE 1960, BEVEGÅRD et al 1960 and 1963, REEVES, GROVER, BLOUNT and FILLEY 1961, WANG, SHEPHERD, MARSHALL, ROWELL and TAYLOR 1961, WILLIAMS, BREDELL, WYNDHAM, STRYDOM, MORRISON, PETER, FLEMING and WARD 1962).

CHAPMAN et al (1960) and WANG et al (1961) have also noted a progressive stroke volume increase during heavier work. WANG et al (1961) summarize their experiments thus: At maximal work, however, there is evidence for a further significant increase in stroke index. The mean value in their study for the largest stroke volume during work (running) surpasses by 10 % that measured while lying down at rest. Unfortunately CHAPMAN et al's (1960) hypothesis is based mainly upon the calculation of the mean; no consideration is taken of the relative load upon the individual. This factor could complicate analysis in the case of the group's being non homogeneous in regard to individual aerobic work capacity. There is an inter individual variation in the size of the stroke volume. Those with a high work capacity are usually distinguishable from those with a low work capacity by a larger stroke volume (CHRISTENSEN 1932 b, MUSSHOF et al 1959, HOLMGREN et al 1960, BEVEGÅRD et al 1963). RUSHMER (1959) on the basis of the data in the literature has suggested that a well trained subject should exhibit an increase in stroke volume during work. MUSSHOF et al (1959) also found a greater increase among the well trained, but neither WANG et al (1961) nor BEVEGÅRD et al (1963) found any difference between trained and untrained subjects in regard to the stroke volume's behaviour during work.

There are large individual variations in the arterio venous oxygen difference even during heavy and maximal loads (ASMUSSEN and NIELSEN 1952, MITCHELL et al 1958, CHAPMAN et al 1960). ASMUSSEN and NIELSEN (1955) pointed out that well trained subjects and groups probably have a hyperkinetic circulation, at least during *submaximal work*; this implies a lower arterio venous oxygen difference at a certain oxygen transport as compared with untrained individuals. A number of studies support this theroy (LINDHARD 1915, CHRISTENSEN 1932 b, JORGENSEN 1954, BEVEGÅRD et al 1963).

BRANNON, MERILL, WARREN and STEAD (1945), and BISHOP, DONALD and WADE (1955) among others, have shown that in anaemia during rest and light work the blood's low oxygen transport capacity is compensated for by an increase in cardiac output. SPROULE, MITCHELL and MILLER (1960)

confirmed earlier results and simultaneously demonstrated that anæmic patients could attain the same cardiac output (23 l/min) as normal subjects during near maximal work Due to the low oxygen capacity of the blood in anæmia, the oxygen uptake was definitely lower (1 8 l/min for anæmic patients versus 3 0 l/min for normals) This sheds some light upon the significance of the hemoglobin content of the blood, but studies are as yet lacking on the meaning of a variation in the hemoglobin concentration within physiological limits for cardiac output during rest and work

Fluid Distribution in the Body During Short Term Muscular Work

A change from rest to muscular work induces an increased hemoglobin concentration and therewith an increase in the oxygen capacity of the arterial blood (for references see HOLMGREN 1956 ch 7) DILL, TALBOTT and EDWARDS (1930) found the hemoglobin increase to be followed by a corresponding increase in plasma proteins From this they concluded that the hemoconcentration was caused by a displacement of fluid from the blood out into the tissues KALTREIDER and MENEELY 1940 EBERT and STEAD 1941, CASSELS and MORSE 1942 verified, through measurement of Evans blue space before and during heavy work that the hemoconcentration was caused by a plasma filtrate overflow to the tissues The size of the overflow was related to the work intensity (see also HOLMGREN 1956 ch 7) That *in the human no non circulatory true blood depots are emptied during work* or upon adrenalin injection has been demonstrated by KALTREIDER, MENEELY and ALLEN (1942) with Evans blue and by NYLIN (1946 and 1947) with tagged erythrocytes The same was done by SJOSTRAND (1948) with COHb method for the determination of total hemoglobin content UEHLINGER and BUHLMAN (1961) arrived at conclusive confirmation of earlier results by simultaneous measurement of erythrocyte (Cr^{51}) and plasma volume (I^{131}) during light muscular work It is worth notice in HOLMGREN s results that the hemoconcentration does not increase beyond the fifth minute of 30 minutes work at 1200 or 1500 kpm/min[1] After work the hemoglobin concentration in the blood returns to the pre exercise value within 60—90 minutes (CULLUMBINE and KOCH 1949)

CULLUMBINE and KOCH (1949) used both Evans blue and thiocyanate for a survey of the body water shift during short term muscular exercise The thiocyanate space increased more than could be explained by the reduction in Evans blue space This may be dependent upon a decrease in the intra cellular space KRONFELD MACFARLANE, HARVEY HOWARD and ROBINSON (1958) whose subjects performed very heavy work in heat used the same

1 kpm = kilopondmeter 1 kp is the force acting on the mass of 1 kg at normal acceleration of gravity 100 kpm/min = 723 foot pounds/min = 16 35 watts

trace substances as did CULLUMBINE and KOCH They found however, no alteration in thiocyanate space while Evans blue space was markedly reduced

Problems

The intention in this section has mainly been to investigate the following problems

a) The effect of work time on the measured value of the maximal oxygen uptake during maximal work
b) Critical size of the involved muscle group for determination of the individual s maximal oxygen uptake
c) The reproducibility of the dye dilution technique and the acetylene method at repeated determination of the cardiac output and the validity of the acetylene method
d) The normal circulatory response during exercise up to maximal level

PRESENTS STUDIES

Material

The results in this section are based upon studies performed on 12 females (f) and 20 males (m) [1 f 4 m (I) 1 f 6 m (II) 11 f 12 m (III) 4 m (IV)] Physical fitness varied from that of sedentary living to that of athletes (n=4) All subjects participated in regular physical activity More detailed information including anthropometric data accompanies each individual study

Methods

The following method and procedure descriptions are done in brief Detailed information is found in the separate reports

Unless otherwise indicated muscular work was performed always in a sitting position on a Krogh bicycle ergometer Heart rate was recorded on an electrocardiograph (Elema Mingograf 42) Expired air was collected in Douglas bags and the volume measured in a spirometer Gas analysis was performed in a modified Haldane apparatus

Lactic acid concentration was determined according to STROM s (1949) slightly modified BARKER and SUMMERSON method (1941) and several samples were drawn to secure the peak concentration (I ÅSTRAND 1960 pp 15—

16) The standard error of a single determination was calculated from 15 duplicate determinations for concentrations between 1 8-14 4 mMol/l (mean 6 3) *and was found to be 2 5 per cent (regarding different levels of lactate see* I. ÅSTRAND 1958) Cardiac output was determined by dye dilution using Cardio green as indicator (III, IV) it was also calculated from oxygen uptake and arterio venous oxygen difference, as described by GROLLMAN (1929) and CHRISTENSEN (1931 a) (IV) Teflon catheters were inserted percutaneously (BERNEUS, CARLSTEN, HOLMGREN and SELDINGER 1954) Certain theoretical objections can be made to the acetylene method (WERKO et al 1949, CHAPMAN et al 1950 JERNERUS LUNDIN and PUGH 1963), but since in one of the studies (IV) the cardiac output measurements were to be repeated a number of times during the course of several months it would have been unrealistic to try to use the method requiring arterial catheters Oxygen content and capacity of arterial blood was determined according to Van Slyke, and hemoglobin was measured spectrophotometrically (BECKMAN B) Plasma volume was determined with Evans blue dye (WIKLANDER 1956), and heart volume was determined in supine position roentgenographically according to JONSELL (1939) Current statistical methods have been used (BONNIER and TEDIN 1957) All significances were tested on the 5 per cent level

Procedure

The duration of submaximal work was about ten minutes, and the measurements were begun after about five minutes There was a short pause between periods Maximal work was always preceded by a warm up period at approximately 50 % of the maximal oxygen uptake Only one maximal work trial took place per day

RESULTS

Determination of Maximal Oxygen Uptake and Heart Rate

Load versus time After a warm up period by exercise with 50 % of max V_{O_2} oxygen uptake and heart rate were recorded continuously from the beginning of very heavy work at various loads until the subjects (n = 5) reached the point of exhaustion Three to five loads were chosen with the intention of exhaustion occuring within two to eight minutes The increase in oxygen uptake and heart rate was greatly accelerated with a larger increase in work load With a work time from two to eight minutes (see Fig 1) the measured oxygen uptake upon exhaustion reached a relatively fixed point for each individual The heart rate exhibited the same pattern as did oxygen uptake Lactic acid at exhaustion was approximately 10 mMol/l or more

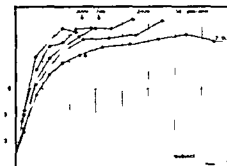


Fig 1 (From 1) Individual curves for one subject showing increase in oxygen uptake ($\dot{V}_{O_2}$) when heavy exercise starts after a 10-min warm up period Arrow marks time when exercise stopped due to exhaustion of the subject Figures indicate work load (x = work could continue for more than 8 minutes)

displaying a tendency toward higher values with heavier work loads Pulmonary ventilation climbed more steeply with heavier loads and reached a definite peak with the heaviest load

Reproducibility of the method In all, 42 experiments were made on the five subjects The difference was calculated in per cent for each subject between the highest oxygen uptake obtained in the actual experiment and the mean of such a maximum in all his experiments The standard deviation of maximal $\dot{V}_{O_2}$ was 3.1 % which includes biological and methodological variables The standard deviation of the maximal heart rate was three beats per minute (according to a statistical analysis of the same type as the preceding)

Oxygen uptake and heart rate versus active muscle mass In order to determine the importance of the muscle group size needed to attain the individual s maximal oxygen uptake the highest oxygen uptake value attained during cycling was compared with that attained during other types of mus

Type of Work	N	Mean $\dot{V}_{O_2}$ liters	Diff	P
Cycling	6	4.23		
Cycling arms + legs		4.24	0.01	0.5
Cycling	5	4.47		
Running		4.69	0.22	0.05
Cycling	6	4.36		
Skiing		4.48	0.12	0.2
Cycling	5	4.47		
Cycling supine position		3.85	0.62	<0.001
Cycling	3	4.66	1.39	<0.001
Cranking		3.27		

Table 1 Comparison between maximal oxygen uptake in cycling in sitting position and other types of muscular work

cular work (see Table 1) Table 1 shows that oxygen uptake during maximal cycling is not significantly different from the maximal oxygen uptake attained with cycling and cranking running or skiing Cycling in a supine position or cranking however yielded definitely lower maximal oxygen uptake values Maximal heart rate was almost the same in all those muscular performances in which there was no difference in maximal oxygen uptake

Cardiac Output During Work

Error of the method No significant difference was found between the acetylene and the dye dilution techniques during rest and work up to the maximal level (see Fig 2) The standard deviation for double determinations of

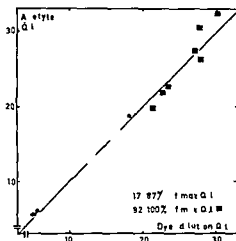


Fig 2 Simultaneous determinations of cardiac output with the dye dilution technique and the acetylene method at rest and during various levels of muscular exercise up to maximal

cardiac output per liter oxygen uptake with *the dye dilution technique* was 4 % at an oxygen uptake of around 1 5 l/min Repeated determinations were made on subsequent days or else with ten minutes interval with the acetylene method and at a constant work load *The acetylene method* showed a standard error of a single determination of 5 9 % at submaximal work loads and 6 4 % at maximal work loads

The relationship between oxygen uptake and cardiac output The circulatory adaptation to work was investigated via the dye dilution method in a group of eleven females and twelve males (III) and via the acetylene method in four males (IV) With the dye dilution method the cardiac output determinations were done in one session only with loads increasing up to maximal With the acetylene method two to four sets of measurements were taken upon three subjects on various occasions during the course of two to five months Regular physical acticity was maintained during t[illegible]
In this publication the two series will be presented [illegible] ce they exhibit nearly identical results In the d [illegible]

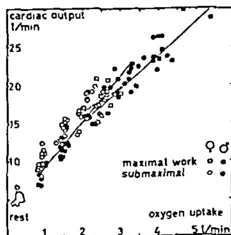


Fig 3 Individual values on cardiac output in relation to oxygen uptake at rest during submaximal and maximal exercise with 23 subjects sitting on a bicycle ergometer Regression lines were calculated for experiments where the oxygen uptake was (i) 20—70 per cent of the individual's maximum and (ii) above 70 per cent Equations

Females (i) $\dot{Q} = 6.13\ Vo_2 + 4.48$ n = 22 SD = 1.23 r = 0.86
(ii) Q = 3.23 Vo + 9.98 n = 26 SD = 1.72 r = 0.58

Males (i) Q = 5.98 Vo_2 + 3.10 n = 32 SD = 1.43 r = 0.95
(ii) Q = 4.35 Vo_2 + 6.55 n = 24 SD = 1.83 r = 0.86

difficult to define accurately the point beyond which the relationship between Q and Vo is no longer linear Too few determinations were made in *this area* The slope of the regression line between cardiac output and oxygen uptake differed significantly, however above and below 70 % of the aerobic capacity (see Fig 3) The breaking point in the second study corresponded to 40 % of the aerobic capacity and the inclination for the two lines were significantly different The equation for the relationship between cardiac output and oxygen uptake was

12—40% of max Vo_2 Q = 6.34 Vo + 4.07, n = 32, SD = ± 0.88 r = 0.94

>40% of max Vo Q = 4.10 Vo + 8.93, n = 95 SD = ± 1.76 r = 0.91

The highest measured cardiac output among the males was 32.5 (C H) 29.9 and 28.1 l/min, with an oxygen transport of 5.45 4.68 and 5.39 l/min respectively The corresponding highest values among the females were 20.9 and 20.7 l/min with an oxygen uptake of 2.67 and 2.35 l/min

In the increase of cardiac output both heart rate and stroke volume (in sitting work position) contributed up to 40 % of the aerobic capacity (Fig 4) The stroke volume showed no significant change past this work level The increase in cardiac output above this level therefore was almost solely a function of the increased heart rate The arterio venous oxygen difference increased gradually during work up to the maximal level If the increase in the arterio venous oxygen difference is related to the relative load of the in

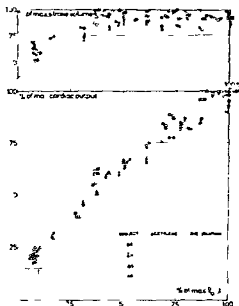


Fig 4 (From IV) Individual values for four male subjects on cardiac output and stroke volume in relation to oxygen uptake All values are expressed as percentage of the highest attained value All determinations are done with the subject sitting on a bicycle ergometer

dividual expressed as percent of the aerobic capacity the relationship is almost linear The resting oxygen content for women was 16 5 ml/100 ml of arterial blood and increased to 17 2 ml during maximal exercise the corresponding figures for men were 19 2 to 19 7 ml The females had at all levels of oxygen uptake a lower arterio-venous oxygen difference than the men A correlation was found between this lower difference and the female s lower oxygen content in arterial blood This implies that the females compensated for the lower arterial blood oxygen content by a larger cardiac output Cardiac output per liter of oxygen uptake averaged 1 1 liters higher for the women than for the men both at submaximal (Vo 1≈1 5) and maximal loads (The difference was statistically significant)

DISCUSSION

The intention in this section (PART I) has been to illustrate the normal pattern of oxygen transport during heavy to maximal work This includes an attempt to appraise the standard question of limiting factors in maximal oxygen uptake (see ÅSTRAND 1952 ch 9 1956 MITCHELL et al 1958, TAYLOR 1960 ch 8) The most important points to which this study may contribute some information are (i) maximal oxygen uptake at the engagement of various large muscle groups (ii) pulmonary ventilation and (iii) the central circulation s transport of oxygen

(i) This study does not agree completely with earlier work since maximal leg work (bicycling in the head up position running) was sufficient to load the oxygen transport system to the maximum and no further increase was noted in combined arm and leg work (CHRISTENSEN and HOGBERG 1950, ÅSTRAND 1952 p 119, TAYLOR et al 1955) If the vascular bed is the limiting factor as suggested by ÅSTRAND (1952 ch 9) the diffusion area of the muscle mass engaged in leg work nevertheless seems to be sufficient to take on the maximum oxygen volume provided by the heart That this is a complex situation to analyze however, is illustrated by the fact that when leg and arm work were combined maximal oxygen uptake and heart rate showed no further increase but work could be tolerated for up to twice as long a time as during leg work alone

(ii) Pulmonary ventilation under normal conditions is not considered a limiting factor, which is supported by the results from Study I The successively heavier maximal loads were accompanied by a higher pulmonary ventilation (increase for the males from mean value 147 to 169 l/min) despite the oxygen transport s remaining constant Thus at the 'lighter maximal load pulmonary ventilation was not limiting but at an increased load and lung ventilation probably a smaller proportion of the total oxygen uptake was made available for the usual work (OTIS 1954) OTIS submitted a pulmonary ventilation of 140 l/min as the critical value during muscular work This probably holds true for many persons but it may be individually set Several of the present subjects regularly demonstrated a pulmonary ventilation of 170 l/min and more at maximal work (bicycling)

(iii) Fig 5 illustrates the relation between aerobic work capacity and the volume of oxygen offered to the tissues (maximal cardiac output x oxygen content of arterial blood) This yields an oxygen utilization coefficient

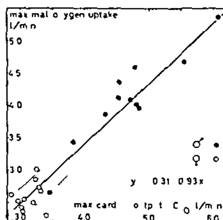


Fig 5 (From III) Maximal oxygen uptake in relation to the volume of oxygen transported from the left heart per minute during maximal work (thin lines denote ± 1 SD)

($\dot{V}o$ x 100/$\dot{Q}$ x CaO) of 83 and 86 % for females and males respectively, and an estimated oxygen content of mixed venous blood of 3 0 ml for females and 2 8 ml/100 ml of blood for males As long as the blood returning to the right part of the heart is not completely devoid of oxygen a further increase in the arterio venous oxygen difference is theoretically possible The estimated values of the oxygen content of the mixed venous blood are so low in this study, however, that it would be unrealistic to count on any further marked increase in arterio-venous oxygen difference The oxygen volume available to the tissues thus becomes limiting for the attained maximal oxygen uptake The cardiac output becomes decisive with a constant hemoglobin concentration (oxygen content of arterial blood)

Conclusions essential to the Continuation of the Study (PART II)

1 Aerobic work capacity can be determined during cycling work inducing exhaustion within two to eight minutes Since working capacity probably is reduced during dehydration a six minute load was chosen as the maximal work (Fig 1)

2 A maximal stroke volume was maintained also in the sitting position at work levels heavier than 40 % of the aerobic work capacity (Fig 4) A load heavier than 40 % of the aerobic capacity (heart rate for young subjects over 120 beats/min) should be used, therefore for any definite evalution of assumed changes in stroke volume during submaximal work Thus 45 and 75 % of the subject s aerobic work capacity was utilized at the submaximal work loads of this study

3 The results yielded by the dye dilution and acetylene methods for the determination of cardiac output agree well during work up to maximal (Fig 2) and reproducibility of the two methods is good

4 The reproducibility of the determination of oxygen uptake, heart rate and lactic acid content of the blood has been investigated at submaximal and maximal work and shown to be high

PART II

BACKGROUND

Physiological Effects of Prolonged Work

A subject experiences increasing fatigue during prolonged work Even when well motivated for the completion of the task the subject will eventually be forced to discontinue due to exhaustion The exhaustion may be extreme and can even cause death (for example, the well trained Pheidippedes after running from Marathon 490 B C) BARGER RICHARDS METCALFE and GUNTHER (1956) have investigated dogs and found that a well trained dog can run to exhaustion and death It is difficult to arrive at a definition of fatigue and exhaustion BARTLETT (1953 p 1) tried

Fatigue is a term used to cover all those determinable changes in expression of an activity which can be traced to the continuing exercise of that activity under its normal operational conditions and which can be shown to lead either immediately or after delay, to deterioration in the expression of that activity

The heart rate with knowledge of the maximal rate (ROBINSON 1938 I ÅSTRAND 1960 Part IV) and the accumulation of lactic acid in the blood during short term work (< 20 min) express the degree of stress rather well (ÅSTRAND 1952 ch 11 ÅSTRAND and RYHMING 1954, HOLMGREN and STROM 1959, BORG 1962) In extended work however, there are no physiological parameters which are perfectly correlated to the fatigue experienced

It is true that the excess lactate (HUCKABEE 1958) starts to increase at 55 % of aerobic work capacity (STRYDOM WYNDHAM RAHDEN and WILLIAMS 1962), but during a prolonged work period blood lactic acid gradually decreases again after the peak concentration has been reached at about 10 to 20 minutes of work This happens even if the blood lactate level is as high as 7—8 mMol/l (BANG 1936) In a study done by HEDMAN (1957) four subjects skied to exhaustion The extreme exhaustion actually experienced by the subjects was not directly expressed in the glucose and lactic acid content of the blood or in the pulse rate The situation was similar in experiments composed of seven hours work at 50 % aerobic work capacity involved (I ÅSTRAND 1960 Part VII)

Thus it is not always possible to define by objective measurements the degree of fatigue involved in extended heavy work A good number of changes (disturbed equilibrium I ÅSTRAND 1960 p 79) do however take place these combined can render a concept of the causation of fatigue

The body's energy kinetics result in a heat surplus (metabolic heat) the size of which is magnified in muscular work In addition, a thermal heat load can occur, depending upon environmental temperature (in regard to temperature regulation see Bazett 1949, HARDY 1961, VON EULER 1961 HERZFELD 1963 Part 3 section VII, LEITHEAD and LIND 1964 ch 3) During muscular work the body temperature is regulated with a thermostat set at a higher level this level in turn is regulated by the relative work load on the individual (NIELSEN 1938, BERGGREN and CHRISTENSEN 1950 I ÅSTRAND 1960 pp 68—70)

The necessity for heat elimination is increased and this is met by, among other things the following mechanisms

(a) *An increase in blood flow through the skin* (EICHNA, PARK, NELSON, HORVATH and PALMES 1950, BISHOP 1956 BISHOP DONALD, TAYLOR and WORMALD 1957, ROBINSON 1963 Part 3 ch 27 in HERZFELD) since the thermal conduction of heat from the 'core to the body surface is not sufficient (see HARDY 1949)

(b) Sweat production the degree of which depends partially upon environmental temperature and heat production (GOSSELIN 1947 ch 4 in ADOLPH, NIELSEN 1938)

(a) During resting conditions exposure to heat causes an increase in cardiac output (30—75 %) which increases the heart rate (among others EISALO 1956 CARLSTEN GUSTAVSSON and WERKO 1961) The pulse rate at a given oxygen transport is higher if the work is performed in a hot environment as compared with a cooler one (ASMUSSEN 1940 BROWN and TOWBIN 1947 ch 12 in ADOLPH CHRISTENSEN 1953, WILLIAMS et al 1962, BROUHA MAXFIELD SMITH Jr and STOPPS 1963) The pulse rate difference between a hot and a cool environment decreases with heat acclimatization (TAYLOR HENSCHEL and KEYS 1943 EICHNA et al 1950) According to WILLIAMS et al (1962) the pulse rate increase is caused by a decrease in stroke volume the cardiac output remaining unchanged (2 heat acclimatized subjects) There should then occur an increase in blood flow to the skin at the expense of the flow to other parts of the body That the excess lactate *was higher in hot environment may be considered proof that the* blood flow to the muscles is lower during work in the 37 degree room than in the 21 degree room If heat production surpasses the maximal quantity which can be given off from the skin per time unit (BURTON 1934 see also SCHOLANDER 1958) there ensues a further rise in body temperature At this point work must either be interrupted or a more temperate environment provided if heat stroke is to be avoided (EICHNA ASHE, BEAN and SHELLEY 1945 CHRISTENSEN 1953, HATCH 1963 and LIND 1963 ch 29 respectively 31 in HERZFELD) Not only a high body temperature but also a high skin temperature can cause heat stroke

(b) Sweating intensity is influenced by many factors (see HARDY 1961 pp 546—550 von EULER 1961 pp 366—368) During work, according to NIELSEN (1938) and ROBINSON (1949 pp 202—212) body and skin temperature play the dominant roles The sweating mechanism however is subject to fatigue Exposure to heavy heat stress GERKING and ROBINSON (1946) found a decrease in sweating rate This occurs wether the skin and body temperature remain constant or rise (PEARCY, ROBINSON MILLER THOMAS and DE BROTA 1956) which may speak for an altered sensitivity to these stimuli (AHLMAN and KARVONEN 1961) GOSSELIN (1947 ch 4 in ADOLPH) however found no decrease in sweating intensity despite a fluid loss of up to 11 % In a group who drank water, on the other hand there was a tendency toward higher sweating intensity

The chemical composition of sweat is determined by several factors such as sweating intensity skin temperature hormones salt content of the body and the diet and degree of acclimatization (see ROBINSON and ROBINSON 1954 KUNO 1956) It should be stressed that a relatively large electrolyte loss can be a result of a non acclimatized person s exposure to too great a heat stress (DILL, HALL and EDWARDS 1938) Since heat cramps can result from low electrolyte concentrations (MOSS 1923 WYNN 1956) the replacement of the electrolyte and fluid deficiency by pure water implies an increased risk (MARRIOTT 1950 p 35)

It is easy to measure a fluid loss incurred during a short time period since body weight changes constitute a satisfactory indicator (ADOLPH 1947 ch 3 pp 36—37) Electrolyte losses are more difficult to quantify Various methods of sweat collection render varying results and in addition the electrolyte concentrations can differ in various parts of the body (ROBINSON and ROBINSON 1954 GRONBAEK 1959) An erroneous calculation of perspiration insensibilis may also play a role but is of minor importance (THAUER ZOLLNER and KAUFMANN 1954) Changes in work capacity raised body temperature etc usually are related therefore to body weight reduction disregarding differences in salt loss

Perspiration is hypotonic in comparison to the body fluids and ought to give a hypertonic dehydration (DARROW, YANET 1935 NADAL PEDERSEN and MADDOCK 1941) The changes in sodium and chloride concentration in blood after a sweat loss are usually small however and they parallel if anything plasma protein changes (ADOLPH 1947 ch 10 SAITO TAKAMATSU 1956 Pearcy et al 1956)

Very few experiments have been done to verify whether or not the above mentioned classical concepts are valid during and after body weight reduction (dehydration) caused by heat load (BASS and HENSCHEL 1956) ADOLPH (1947 ch 10) found a reduction in Evans blue space after light work in a hot environment The individual variation was large but there was a correlation between dehydration and the reduction

of plasma volume The contraction of the Evans blue space was a good deal larger (2 5 times) than could be predicted from the plasma volume s proportion of the body s total fluid content These studies have been used as a standard, and in later investigations ADOLPH s data has often been used directly in order to predict the blood volume decrease in dehydration (BEETHAM and BUSKIRK 1958) A large reduction in Evans blue space accompanied by relatively small changes in electrolyte concentrations does not agree completely with hypertonic dehydration For a discussion of the regulation of blood volume in general, see GREGERSEN and RAWSON (1959) SJOSTRAND (1962) neuroendocrine aspects FARRELL and TAYLOR (1962) volume receptor aspects GAUER and HENRY (1963) For a more complete outline of fluid balance see for example ELKINTON and DANOWSKI (1955) BLAND (1956)

Dehydration with a gradual decrease in the circulating blood volume produces a further load upon the circulatory apparatus partially comparable to the effect of a withdrawal of blood (BALKE GRILLO, KONECCI and LUFT 1954, GULLBRING HOLMGREN SJOSTRAND and STRANDELL 1960) A gradual dehydration also causes in the supine position a decrease in stroke volume which is compensated for by a rise in the pulse rate (ROTHSTEIN and TOWBIN 1947 ch 11 in ADOLPH) With a change in position (standing passive tilting) the circulatory regulation is made more difficult (ASMUSSEN CHRISTENSEN and NIELSEN 1939 a and b, LAGERLOF ELIASCH, WERKO and BERGLUND 1951, SJOSTRAND 1953) After dehydration the pulse rate increase in upright position is more definite (BEETHAM and BUSKIRK 1958), and syncope may occur after a short period in the head up position (ROTHSTEIN and TOWBIN 1947 ch 11 in ADOLPH) After dehydration there is also an exaggerated heart rate response to standardized muscular exercise (PITTS JOHNSON and CONSOLAZIO 1944 BEAN and EICHNA 1943, BUSKIRK, IAMPIETRO and BASS 1958) The rise in pulse rate above and beyond that required by the energy load is related to the degree of dehydration (BROWN and TOWBIN 1947 ch 12 in ADOLPH) On the basis of this BROWN and TOWBIN tried to estimate the decrease in physical work capacity during gradually increased dehydration

During heavy heat stress the blood flow through the skin is not always sufficient to offset the heat production When dehydrated the problem is aggravated by any gradual decrease in both circulating blood volume and sweating intensity Therefore the body temperature as well as pulse rate, is a good indicator of the individual tolerance to an increasing dehydration both at rest and during work this under the stipulation that one knows what the relationship is under normal conditions, between pulse rate rectal temperature, and oxygen uptake Both parameters (pulse rate and rectal temperature) can be utilized as objective criteria for assessment of the effects of acclimatization (e g BEAN and EICHNA

1943, Adolph 1947, Buskirk et al 1958) In regard to the physiological adjustment to heat, see Conn (1949 and 1963) Bass Kleeman Quinn Henschel and Hegnauer (1955) and Bass (ch 28 in Herzfeld 1963) An evaluation of the effect of water and salt administration during dehydration can be made with these criteria also (Bean and Eichna 1943 Pitts et al 1944, Ladell 1955, Pearcy et al 1956) Pulse rate and body temperature measurements thus can be used as indicators of the circulatory load and heat balance but it is considered impossible to make an exact quantitation of the decline in physical work capacity Thus the methods of measurement are limited and therefore there is still a lack of knowledge of the factors which regulate and limit physical work capacity during prolonged work

Available Energy Depots

Protein plays a completely non essential role as a source of energy during muscular work (Pettenkofer and Voit 1866, Margaria and Foa 1939) but both fat and carbohydrates can be utilized The proportion for carbohydrates rises with increasing work intensity (Bock et al 1928 b Christensen 1932 a Edwards Margaria and Dill 1934 Margaria 1939) During prolonged work the carbohydrate contribution decreases gradually as a source of energy but the blood sugar concentration does not invariably drop to a hypoglycemic level (Christensen 1931 c Boje 1936 Christensen and Hansen 1939 a Hedman 1957) In these studies exhaustion followed after work for 1 1/2 hrs or more and the combustion of 200 to 450 gm of carbohydrates (estimated from oxygen uptake and respiratory quotient) In most cases they observed low blood sugar values and symptoms of hypoglycemia Light muscular work (Dill, Edwards De Maeio 1935 fasting walk Gothenburg — Stockholm 500 km 1954) but not heavy can be performed in the presence of greatly reduced glycogen depots (Christensen and Hansen 1939 a b Hedman 1957) Administration of adrenalin (i m) facilitates the increased utilization of carbohydrates (Dill et al 1935)

A subject exhausted as a result of reduced glycogen depots can resume work after the administration of glucose (Christensen and Hansen 1939 b c) This is also true in dogs as is nicely illustrated in the case of the dog Joe (Dill Edwards and Talbott 1932)

The limitation of the ability to utilize fat as fuel during hard muscular work is considered by many to depend upon quantitatively deficient mobilization from the depots A rapid and large mobilization of fatty acids occurs during work (Friedberg Harlan Trout and Estes 1960 Carlson and Pernow 1961 Carlsten Hallgren Jagenburg Svanborg and Werko 1962 Issekutz 1964) During extended work this mobilization increases gradually (Basu, Passmore and Strong 1960 Carlsson Eke-

LUND and ORO 1963, SALTIN 1964) Thus the situation seems to be complex and for a more complete discussion of fat as fuel see for example FRITZ (1961) and RANDLE GARLAND, HALES and NEWSHOLME (1963)

One result of the increased fat consumption is that the mechanical efficiency decreases by up to 10 % (KROGH and LINDHARD 1920, CHRISTENSEN and HANSEN 1939 b see also P O ÅSTRAND 1956) Thus the rise in pulse rate which occurs during extended work can be explained to a small extent only by a rise in oxygen consumption The question is, then, whether or not the increased blood flow to the skin and increasing dehydration are those fators which explain the residual rise in pulse rate, or if the altered metabolism in itself influences the regulation of heart rate during work

During acute starvation (four days) there occurs a gradual decrease in the physical work capacity (HENSCHEL, TAYLOR and KEYS 1953) Noticeably enough, however, the maximal oxygen uptake did not decrease by more than about 7 % After semi-starvation (six months) the decrease in physical work capacity became more marked and there was a 25 % decrease in aerobic work capacity (KEYS BROZEK HENSCHEL MICKELSEN and TAYLOR 1950)

Problems

This summary leaves many questions open The intention in this section has been to define the effect of dehydration (sweat loss) upon

(a) Body fluid compartments particularly the blood volume
(b) The hemodynamics of submaximal and maximal work
(c) Physical work capacity estimated from work time at a maximal load

PRESENT STUDIES

Material

The group was composed of 6 females (f) and 50 males (m) in the various series [3 m (IV) 14 m (V) 6 m (VI) 6 (f) and 22 (m) (VII) 10 m (VIII) 4 m (IX)] All were healthy adults Certain of the subjects were champion skiers, that is all females and 2 and 18 of the males in studies V and VII respectively The remaining subjects work capacity and degree of training varied from sedentary living up to athletes (n=2) All subjects were unacclimatized to heat More detailed information including anthropometric data can be obtained from the separate studies

Methods

In regard to the determination of oxygen uptake cardiac output heart rate hemoglobin concentration and lactic acid in the blood and statistics

are referred to Part I p 13 Body weight was measured to an accuracy of ±50 gm Plasma volume was determined with Evans blue dye (T—1824) according to VON PORAT (1951) and WIKLANDER (1956) Standard error of a single measurement, determined from 12 double determinations with a week's interval was 2 4 % The blood volume (actually plasma volume) was measured with I^{131} tagged albumin (RISA) according to WILLIAMS and Fine (1961) (SE ± 2 9 % EKELUND and HOLMGREN [1]) The red cell volume was determined with Cr^{51} tagged red blood cells (MOLLISON and VEALL 1955) The heamatocrit was determined with a high speed micro-heamatocrit centrifuge (HAWKSLEY) corrected for trapped plasma by a factor of 0 98 (GARBY and VUILLE 1961) Plasma proteins as well as non protein nitrogen in the urine was determined by KJELLDAHL's macro method Sodium and potassium was determined with the aid of an EPPENDORF flame photometer and chloride according to LEHMAN (1939) The thiosulphate compartment (CARDOZO and EDELMAN 1952) was used for the estimation of the apparent inulin space (IKKOS LJUNGGREN LUFT and SJOGREN 1956) Tritium space was determined according to PRENTICE SIRI BERLIN HYDE PARSONS JOINER and LAWRENCE (1952)

Haptoglobin was determined according to JAYLE (1951) and free hemoglobin in the serum according to CROSBY and FURTH (1956) Blood sugar was determined with ortho toulidine according to HULTMAN (1959) Rectal temperature was measured with a mercury thermometer Enviromental temperature was measured with an Assmann psychrometer The isometric muscle strength was measured with a strain gauge according to TORNVALL (1963) Blood pressures were transmitted to a strain gauge manometer (Elema Sweden EMT 490) connected to an amplifier unit (see HOLMGREN ch 1 1956) Tracings were recorded on a Honeywell Visicorder (galvanometer V 100—350 B) Mean pressures were obtained by electrical integration Work time at a maximal load was utilized for the evaluation of physical work capacity

Procedure

The tests were always performed on a Krogh bicycle ergometer in a sitting work position at room temperature (approx 20° C relative humidity 50 %) unless otherwise stated During the prolonged exercise periods a treadmill and a mechanically braked bicycle ergometer (VON DOBELN 1954) were used interchangeably The general procedure was essentially standard in studies IV VI, VIII and IX The subjects drank 0 5 to 1 0 liters of water extra on the evening previous to an experiment The subjects came to the laboratory in the morning after a light breakfast (normal conditions) and thereupon the various determinations were performed

1 By kind permission from Dr Holmgren and Dr Ekelund their Volemetron could be used in this study

There then followed a heat stress period of from two to four hours induced either by (i) intermittent subjection to a sauna (80° C, rel hum 15 %) (T) or (ii) hard work (around 75 % of max Vo) at room temperature (around 20° C, rel hum 50 %) (E) or (iii) lighter work (around 50 % of Max Vo) at 36—40° C (rel hum 35 %) (E+T) 60 to 90 minutes after the completion of the dehydration period (rectal temperature returned to under 37 3° C), the measurements were repeated (room temperature 20° C) After arriving at the laboratory the subjects ingested neither food nor water until after the completion of the final measurements in the afternoon

Body Fluid Compartments After Dehydration (Sweat Loss)

The conclusions are based mostly on Study VI in which six males were dehydrated through (i) exposure to heat (T), (ii) hard exercise (E), and (iii) lighter work in heat (E+T) on various occasions to about the same degree of dehydration In regard to plasma volume, the results in Studies IV (thermal heat load) and IX (metabolic heat load) have been used also Study V deals also with the changes in plasma and red cell volume in connection with dehydration but since the subjects in that study drank water, the results will be treated separately

RESULTS

Plasma Volume Fig 6 shows that the reduction in Evans blue space is related to the decrease in body weight The individual variation is great, with a resultant wide range It is evident, however, that at the same degree of dehydration Evans blue space is more greatly reduced after the thermal heat load than after the metabolic heat load In an exercise dehydration of 4 % of the body weight the mean Evans blue space was decreased by only 3 % compared with 18 % in thermal dehydration Dehydration caused by combined thermal and metabolic heat resulted in a contraction in Evans blue space which lay between the results from the single factor experiments (at 4 % weight decrease approximately 9 %)

When fluid (mainly water and sugar) was administered during hard cross country skiing over 85 km (V) Evans blue space increased by 0 4 l (range 0 1—0 7 for 6 subjects) this was in spite of the fact that the body's total fluid content was still reduced and the body weight loss was up to 5 5 %

Red Cell Volume In a similar experimental situation (V) red cell volume was measured before and after 6—10 hours skiing The decrease in red cell volume was non significant with a mean value of 0 08 l (range

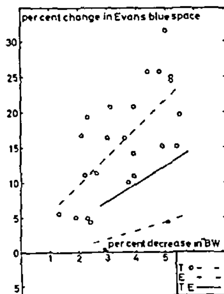


Fig 6 The relation between per cent change in Evans blue space and per cent decrease in body weight (B W) Equation for the regression lines are
Thermal dehydration (T) y = 3 99 x + 1 76 n = 23 SD = 5 6 r = 0 68
Exercise (E) y = 1 24 x — 1.77 n = 16 SD = 4 0 r = 0.31
After exercise in heat (E + T) there were too few values (six) for statistical treatment

0 05—0 17 n=5) Neither haptoglobin nor free hemoglobin in the serum indicated any definite hemolysis

Interstitial Space The changes in apparent inulin space and Evans blue space followed the same pattern The error of the method in the determination of the apparent inulin space is of such a magnitude that an estimation of the interstitial space is of only limited worth There is a tendency however toward a slighter contraction of the interstitial space in comparison with the Evans blue space after sauna dehydration (E) On the other hand, the proportional decrease in interstitial space and in Evans blue space is about the same after dehydration induced by light work in heat (E+T) This holds true for exercise dehydration also but the absolute volume changes are very small (see Table 2)

Intracellular Space The calculated reduction in intracellular space (total fluid loss corrected for the reduction in the apparent inulin space) was 2 9 l after the exercise dehydration compared with 1 7 l after the thermal dehydration which amounted to 96 and 53 % respectively of the total fluid loss in the actual situations The percentual reduction in the calculated intracellular space was after the exercise dehydration 8 4 % compared with 4 7 % after the thermal dehydration (see Table 2)

TBW			ICF			ECF			PV		
T	E	E+T	T	E	E+T	T	E	E+T	T	E	E+T
Distribution of water loss liter											
3 1	3 1	3 5	1 7	2 9	2 2	1 4	0 2	1 3	0 72	0 10	0 40
SD diff = 23			SD diff = 71			SD diff = 86			SD diff = 32		
p>0 7			p<0 001			p<0 001			p<0 001		
Distribution of water loss, per cent											
100 0	100 0	100 0	52 6	96 3	61 8	47 4	3 7	38 2	22 7	2 8	11 4
			SD diff = 32 2						SD diff = 9 1		
			0 01>p>0 001						p<0 001		
Decrease in per cent of initial volume											
6 5	6 5	7 4	4 7	8 4	4 5	11 1	1 2	10 5	18 3	1 5	10 6
SD diff = 52			SD diff = 2 3			SD diff = 6 6			SD diff = 9 2		
p<0 7			p<0 001			0 01>p>0 001			p<0 001		

Table 2 Average values standard deviation (SD) and p value for the intra individual differences in the distribution of the water loss (in liter and per cent) and the decrease of the initial volume of total body water (TBW) intra cellular fluid (ICF calculated value) apparent inulin space (ECF) and Evans blue space (PV) after the three dehydration periods (hot environment = T exercise = E exercise in hot environment = E + T) The significance test referred to the difference between T and E (n = 10)

DISCUSSION

Few comparable studies have been done involving the measurement of the body's various fluid compartments after acute dehydration (sweat loss) ADOLPH s (1947 ch 10) standard data agree well with those given above in regard to plasma volume reduction after work in a hot environment Also ROBINSON (1949 p 209) found a 13 % increase in plasma volume for well hydrated workers in heat A large salt loss through sweating can induce a marked reduction in extracellular space Electrolyte concentration in sweat averaged 80 and 70 mEq/l for sodium and chloride respectively Since all subjects were unacclimatized to heat, and in addition sweating intensity was high a high salt concentration in the sweat could be expected even though in this case the method of sweat collection (impermeable plastic bag around left fore arm and hand) may have contributed to the high salt concentration (ROBINSON and ROBINSON 1954) There was no definite difference in electrolyte loss (Na^+ Cl^-, K^+) among the three types of dehydration The loss of sodium was 25 mEq greater during thermal dehydration, and 5 mEq more potassium was lost during exercise dehydration these differences, however, together with the

small electrolyte displacements within the body (e g the increase in potassium concentration in the plasma during work from approximately 4 0 to 5 5 mEq/l) explain only to a very minor degree the observed differences in Evans blue space

The stored glycogen is consumed gradually during work The water which was previously bound to the glycogen is then liberated (Bland 1956 p 365) This fluid quantity together with the water produced when carbohydrates and fat are combusted should be quantitatively important in providing for sweating without true dehydration (compare SCHMIDT-NIELSEN 1962) It is impossible to make an exact determination of the glycogen combustion An estimate made from oxygen uptake and respiratory quotient during exercise dehydration shows that approximately 3 100 calories are turned over and that approximately 350 gm glycogen are consumed The hypothetical water volume then liberated should total 1 1 l (PUCKETT and WILEY 1932 FENN 1939) Even if this volume (1 1 l) is taken into account there remains a somewhat skewed distribution with a relatively higher loss from the intracellular space during the exercise dehydration (see Table 2) since the true water loss can be estimated to be some two liters With an even distribution of the true water loss the reduction in extracellular space should be 0 6 liters as compared with the observed 0 2 liters It is possible that the rise in plasma potassium during work is not attributable to the glycogen consumption even if the potassium is deposited together with glycogen (FENN 1939) A glycogenolysis induced by i v glucagon administration is not accompanied by any prolonged potassium liberation (FINDOR and HULTMAN 1962) Neither is it probable that there is an increased breakdown of intracellular protein with an outflow of potassium since non protein nitrogen in the urine is not increased during and after work LAURELL and PERNOW (1964) however, among others have demonstrated a certain release of potassium from muscle during the contraction phase (see also KJELLMER 1961 and HULTMAN and BERGSTROM 1962) this most probably explains why the highest potassium content in the blood of normal subjects has been observed in connection with extended muscular work With a lowered osmolarity of the muscle as well as a decrease of glycogen content in the liver and muscles it is possible that a fluid loss caused by sweating occurs mostly at the expense of the intracellular space It is not known whether this surplus fluid quantity is sufficient or if an active regulation intervenes to maintain the blood volume when heavy work is performed for instance hormones (CLINTON and THORN 1943) Volume receptors have been demonstrated in the heart and vessels (GAUER and HENRY 1963) but if they were contributory the present results would imply different responses to a receptor activity during rest (sauna dehydration) and during muscular work with intensive sweating as a result

Effects of Dehydration on the Circulatory Reaction to Submaximal and Maximal Work

This section of the study was prompted mostly by findings at ski races (VII). In spite of the intensive struggle against the clock among cross country champion skiers, only very low concentrations of lactic acid could be demonstrated in the blood after a competition which lasted for an hour or more. Neither was the lactic acid concentration raised by standardized maximal work on the bicycle after the completion of a competition. There was, on the other hand, a surprisingly small decrease in maximum oxygen uptake (ca 10 %) but at marked decrease (ca 30 %) in the maximal load which the subjects were able to tolerate for about three minutes. Under laboratory conditions therefore, ten males were investigated for changes in aerobic and anaerobic work capacity after hard muscular work. This was compared with a corresponding dehydration caused by exposure to heat (T).

The circulatory acclimatiaztion to work was investigated in three males after thermal dehydration (T) (IV) and during and after extended work (E) in four males (IX). In study IV the circulatory reaction to work was investigated before and 90 minutes after exposure to heat. By varying the duration of the heat exposure there was attained a reduction in body weight of from 1.1 to 5.2 %. In study IX additional circulatory measurements were made at intervals during a three hour work period.

RESULTS

Submaximal Work in Upright Work Position

Oxygen Transport. No definite difference could be demonstrated in oxygen uptake during submaximal work before and after exercise and thermal dehydration. There was a tendency, however, toward a small increase in oxygen uptake when the test work took place after exercise dehydration. RQ was lower also.

Heart rate. After both types of dehydration the heart rate in submaximal work was significantly higher than before dehydration. At the same degree of dehydration however, the heart rate was significantly higher after exercise dehydration as compared with thermal dehydration. A 3.6 % dehydration induced by work produced in 10 subjects a mean heart rate of 138 beats per minute at the lighter load (45 % of max Vo) compared with 123 beats per minute before exercise dehydration. Similar measurements made at a 3.8 % thermal dehydration yielded 129 beats per minute as compared with 122 beats per minute.

Stroke Volume. Subsequent to the thermal heat load there was a signi-

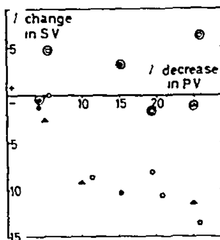


Fig 7 (From IV) Change in stroke volume (SV) during exercise performed after various degrees of dehydration causing a decrease in plasma volume (PV) Circled symbols denote determinations during maximal work those without circles refer to the 45 % load" The equation for the regression line for relative decrease in plasma volume and the change in stroke volume on the 45 % load is y = 0 6 + 0 5 x with a correlation coefficient of 0 87 (p <0 001)

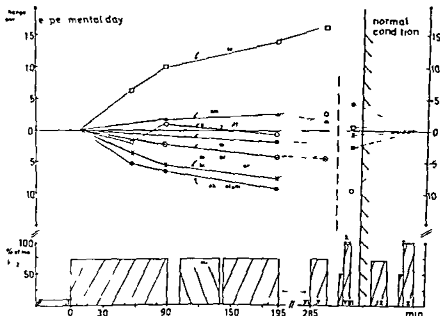


Fig 8 (From IX) *Lower part* Procedure for the experiments both for the experimental day and during normal conditions The effective work time during the prolonged work period was 180 min The Roman figures mark when the measurements were performed *Upper part* The percentual changes in heart rate plasma proteins arterio venous oxygen difference blood volume body weight mean arterial blood pressure and stroke volume during the exercise Notice that for the submaximal work (75 % load) the 5—15 min values (II) represent the zero point and for the maximal work the values obtained under normal conditions are zero (X)

ficant correlation between the degree of dehydration and the reduction of the stroke volume during submaximal work (r=0 65) This correlation was closer at the lightest submaximal load (see Fig 7) During extended work there occured also a gradual decrease of the stroke volume (see Fig 8) This was most marked at the resumption of work after rest when it averaged 15 ml (= 11 % body weight reduction 4 5 %)

Comparison Between Submaximal Work in Recumbent and Upright Work Position

Before and after a thermal dehydration of approximately 4 % 3 subjects worked in both sitting and recumbent positions at a submaximal load (ca 60 % of max Vo) There was hardly any difference in oxygen uptake before and after dehydration The heart rate was 9 7 beats per minute higher in the sitting work position after dehydration but remained constant in the recumbent work position

Before and after extended work, two of the four subjects worked also in the recumbent position After prolonged exercise the trend was almost the same in the hemodynamic response to the submaximal load in both work positions there was a large heart rate increase but practically the same cardiac output as before the period of extended work The stroke volume was reduced by 18 and 34 ml (18 %) respectively in the supine work, and in the sitting work position by 12 and 37 ml (19 %) The body weight reduction in these two subjects was 4 1 and 4 0 %

Maximal Work in Upright Work Position

Measurements of oxygen uptake arterio venous oxygen difference heart rate and therefore cardiac output and stroke volume gave almost identical results under normal conditions and after thermal dehydration The most well trained of the subjects could not attain a maximal load after extended work For the other three the recorded values for oxygen uptake were a few percent lower, and those for the cardiac output were a few percent higher after extended work than after the normal run This was accompanied by an average decrease of 9 % in arterio-venous oxygen difference The highest recorded heart rate after extended work departed very little from the normal maximal heart rate (+ 1 5 beats/min) this implies a small if any tendency toward a larger stroke volume at maximal work after prolonged work as compared with that attained at maximal work under normal conditions The relation between and the changes in the recorded parameters after thermal dehydration are illustrated in Fig 9 and for exercise dehydration in Fig 8

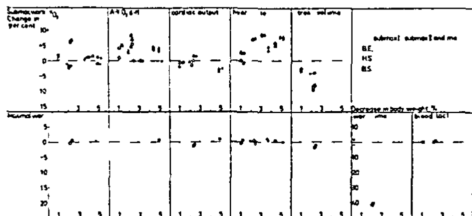


Fig 9 (From IV) Per cent change in oxygen uptake ($\dot{V}o$) arterio-venous oxygen difference (A Vo diff) cardiac output heart rate and stroke volume (ordinata) on two submaximal (upper part) and one maximal work load related to the degree of body weight reduction (abscissa) For the maximal work load data on per cent changes in work time and blood lactates are added and also related to decrease in body weight notice the different scale on the ordinata

Work time at the maximal load was significantly reduced after both thermal and exercise dehydration Direct comparison between the two types of dehydration showed a significantly larger reduction after exercise dehydration as the postdehydration work time averaged 3 2 minutes at the maximal load as compared with 5 0 minutes after thermal dehydration The shorter work time was accompanied by a considerably lower lactate level in the blood This lactic acid concentration was practically the same as that attained when maximal work under normal conditions was terminated at about the same time tolerated by the subjecs after dehydration

DISCUSSION

When under normal conditions muscular exercise is performed in a sitting position a redistribution of blood takes place in a central direction with a subsequent increase in stroke volume (see Part I p 17) With a further increase in the work level above 40 % of the individual s aerobic work capacity, the stroke volume remains constant (Fig 4 p 18) After a thermal dehydration with a reduced circulating blood volume this adjustment to submaximal loads is modified At the lighter submaximal load (i e 45 % load) the decrease in stroke volume was strongly correlated to the decrease in plasma volume At the heavier load (i te 77 % load) a smaller stroke volume was still observed but at maximal work the regulatory mechanisms can at least for a few minutes, com

pensate for a reduced blood volume, and the same maximal stroke volume was attained as under normal conditions In the recumbent work position, thermal dehydration did not result in an increased heart rate, which illustrates the hydrostatic component in the heart rate rise observed after dehydration The experiments in the recumbent position compose a good control study indicating that the extended exposure to heat *per se* did not influence the hemodynamics during muscular work This is supported also by the fact that the four subjects who during exposure to heat kept the body weight constant by drinking saline solution did not have an elevated heart rate at submaximal work after the sauna

Thus, the following mechanisms are likely to be involved in the circulatory adjustments to work in the sitting position after thermal dehydration when changing from a supine to a sitting position the central pool of blood is diminished (SJOSTRAND 1953, HOLMGREN and OVENFORS 1960) After a dehydration reducing the circulating blood volume, the reduction in the central pool of blood is probably even more pronounced At rest and during mild muscular exercise the rate of venous return does not permit a complete filling of the ventricles As the work becomes heavier or maximal, the muscle pump becomes more effective and the level of vaso motor activity is higher, especially on the capacitance vessels (FOLKOW 1956) Thus a redistribution of the blood volume enables the subject to attain a normal stroke volume and cardiac output despite the reduced blood volume

During and after extended work there was no marked reduction in the circulating blood volume or at most 5 % Thus, the gradual decrease in stroke volume during work can not be explained directly by the same hypotheses as after thermal dehydration One possibility is however, that the central pool of blood is reduced by the extended work causing an increased displacement of blood to the periphery of the blood vessel system s low pressure part (EKELUND and HOLMGREN 1962) The finding of a reduced stroke volume also at submaximal loads in the supine position after extended work contradicts this hypothesis however Neither does a change in body temperature seem to influence the results since stroke volume was, if anything still more reduced during submaximal work with a comparatively lower rectal temperature (i e after 90 minutes rest subsequent to extended work)

Thus it is difficult to find an explanation for the successively altered hemodynamic response during extended work at a constant submaximal load Further investigations may show whether or not factors which altered the interior milieu (cell milieu) or altered the production of or sensitivity to catecholamines contribute to the gradual rise of the heart rate Therefore the gradual increase in heart rate is probably not due to a failure of the heart muscle as such to attain a high stroke volume It must be strongly stated however that even after prolonged exercise as

well as after thermal dehydration the regulatory mechanisms during maximal work can maintain at a maximal level, at least for a couple of minutes the cardiac output stroke volume and arterial pressures

During prolonged exercise the most well trained subject s cardiac output decreased throughout the work period This can be interpreted as the well trained subject s circulation being hyperkinetic (Vo 3 4 l—Q 24 5 l) in the beginning of the prolonged exercise and thereafter gradually becoming normokinetic (Vo 3 5 l—Q 22 5 l)

This same subject was the only one out of thirteen who was unable to stand the maximal load after the prolonged exercise period This may be a case in which a reduction of the glycogen depots provides an explanation (CHRISTENSEN and HANSEN 1939 c HEDMAN 1957), even if the blood sugar was not below 75 mg/100 ml since at least 1 600 of 3 100 (from V_{O_2} and RQ) converted calories were derived from carbohydrates

A simple explanation for the decreased work time at maximal work and the concommitant low blood lactic acid after dehydration may be psychological factors which are always decisive during exhausting work However the author himself was a subject and furthermore he is strongly convinced that the other subjects also did their best The subjects usually complained of the legs as being the limiting factor at the maximal load normally as well as after dehydration The shorter work time may thus imply a decreased physiological ability to perform hard muscular work

GENERAL DISCUSSION

When large muscle groups are active for a minute or longer aerobic processes are decisive for continued uninterrupted work (HILL and LUPTON 1923 ÅSTRAND 1956) Oxygen transport engages both the pulmonary and the circulatory systems In many conditions involving decreased work capacity the natural explanation would seem to lie in the oxygen transport links Adequate therapy demands an understanding of the physiological mechanisms responsible for the work capacity decrease

The scope of the present investigation has embraced the analysis of exercise adaptation up to maximal work First under normal conditions to define the normal pattern and secondly after dehydration induced by prolonged work or exposure to heat

Maximal oxygen uptake determinations demand the activity of a large muscle mass throughout a sufficient time period Leg work such as bicycling is usually enough and if the work time lies between two to eight minutes the work intensity becomes crucial for the attainment of a maximal oxygen uptake During lighter work under normal conditions in sitting work position both stroke volume and heart rate contribute to the cardiac output increase Although at a work load of over 40 % of

the aerobic work capacity the heart rate is almost the sole contributor (Fig 4) This retards the rate of increase in cardiac output as the oxygen uptake approaches maximum It may be advantageous to choose a test load which corresponds to at least 40 % of the maximal oxygen uptake The heart rate then climbs above 120 beats/min, and the stroke volume has usually become maximal The importance of the blood hemoglobin concentration in regulating cardiac output is illustrated by the fact that the women at submaximal work compensated for the lower oxygen content of arterial blood by a hyperkinetic circulation After the thermal dehydration which contributed to hemoconcentration there occured a small but significant decrease in cardiac output at submaximal work

ADOLPH and associates (1947) have defined the classical picture of those factors which during sweat loss dehydration cause a decrease in physical work capacity According to ADOLPH, dehydration results in an increased heart rate reaction at submaximal work since stroke volume is lowered due to the quite extreme reduction in blood volume relative to fluid loss This standpoint is supported by the positive effects of fluid administration

The results presented in Part II show also that the hemodynamic response to submaximal work is altered after dehydration However, only after heat dehydration is it possible to correlate the stroke volume decrease incurred during work to the reduction in Evans blue space This applies in the sitting work position only since no change was noted in heart rate during work performed in the supine position after dehydration Since the reduction in Evans blue space after work dehydration was small and the stroke volume reduction was marked in the supine position also neither volume changes nor the displacement of blood volume seem to play a crucial role for the large rise in heart rate at submaximal work after dehydration A drastic illustration of the role of volume changes was found after the ski competition when the post competion blood volume was actually somewhat larger, but during the performance of standard work the heart rate was 15 beats/min higher than normal

In a large number of experimental situations such as acute starvation (HENSCHEL et al 1953), the administration of anticholinergic substances (for references see BEVEGÅRD 1963 ROBINSON PEARCY, BRUECKMAN NICHOLAS and MILLER 1953) dehydration (BROWN and TOWBIN ch 12 in ADOLPH 1947) blood loss (BALKE et al 1954, GULLBRING et al 1960), pyrogen induced fever (GRIMBY 1962 GRIMBY and NILSSON 1963) and heat exposure (WILLIAMS et al 1962) the submaximal work pulse reaction is higher than normal dependant upon either a decrease in stroke volume and/or an increase in cardiac output The studies in which maximal work was also performed show that there usually are small differences in the recorded maximal values of heart rate and oxygen uptake between normal conditions and the experimental situation (HENSCHEL et al 1953,

ROBINSON et al 1953 GRIMBY 1962) This is also true for maximal cardiac output during heat exposure (WILLIAMS et al 1962) The marked hemodynamic changes at the submaximal level which are practically normalized at the maximal level agree with the results of this study (Part II) The maximal level of cardiac output is surprisingly constant in the various experimental situations as compared with normal conditions (Part II, WILLIAMS et al 1962) It is unknown whether there is one regulatory mechanism or several which explain the increased heart rate at submaximal work in the above mentioned situations Caution is necessary, however in these situations in the prediction of aerobic or physical work capacity from the heart rate response to submaximal work

Work time at the maximal load is significantly shortened after dehydration particularly after work dehydration Of those factors noted in the introduction as essential to physical work capacity, motivation (psychological factors) and mechanical efficiency (technique) may be excluded as possible causes for this shortening since they did most likely not alter in connection with dehydration The maximal isometric strength was not reduced after dehydration This may mean that neither the impulse traffic to the muscle nor the muscle s ability to respond undergoes any great change during dehydration There remains the energy output which normally engages both the aerobic and anaerobic processes at maximal work between two to eight minutes After dehydration however the central circulation can distribute a maximal volume of oxygen to the tissues The very low blood lactic acid level at maximal work is striking and it seems logical to submit the aerobic processes as a limiting factor for physical performance after dehydration In the studies (IV, VIII IX) in which a constant maximal load was used an almost constant blood lactic acid level was maintained however, if the subjects worked for the same time at this load both under normal conditions and after dehydration

Fig 10 illustrates this relationship after the ski competition (Vasaloppet 85 km VII) In this study the standardized work was performed so that the work load was increased step wise until the subject reached exhaustion Both the work intensity and the blood lactic acid concentration thereby attained were greatly reduced in comparison with normal conditions For the present therefore it is impossible to decide whether decreased anaerobic capacity primarily limits work capacity, or if it is some other factor inhibiting the muscles capacity for dynamic contractions

The decrease in physical work capacity after dehydration has been assayed either from the reduction in work time at a fixed maximal load or from the decrease in maximal work load tolerable for a certain period of time The effect of these changes on the recorded values for oxygen uptake and blood lactic acid is illustrated in Fig 11 A decrease in maximal work time (Fig 11 A) from 5 5 to 3 5 minutes implies that the oxygen

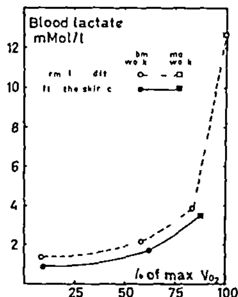


Fig 10 Peak blood lactate concentration after submaximal and maximal exercise during normal condition and after a ski race (n=5) Notice that the values of the abscissa are presented as per cent of the maximal oxygen uptake during normal condition

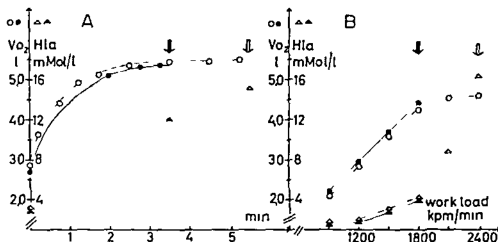


Fig 11 A Oxygen uptake and peak blood lactate concentration at a maximal work load which could be maintained for 5.5 min during normal conditions (unfilled symbols) and 3.5 min after dehydration (filled symbols)
Fig 11 B Oxygen uptake and peak blood lactate concentration at submaximal and maximal work loads during normal condition (unfilled symbols) and after dehydration (filled symbols)
Arrows mark maximal work time and work load respectively

transport can be accelerated practically up to the maximal level but that a maximal concentration of lactic acid is not accumulated. At a reduction in the maximal work load which was tolerated for a period of six minutes (Fig. 11 B) there arose an analogous situation in which the 25 % decrease in work load from 2400—1800 kmp/min caused a decrease in oxygen uptake from 4.55 to 4.35 l/min while the lactic acid concentration in the blood decreased from ca. 15.5 to 4.0 mMol/l.

Dehydration through perspiration is not uncommon in Sweden in spite of the country's northerly location. Metabolic heat is usually the predominant heat stress factor but in industry, for example, there are many occupations with a high environmental temperature (VII, CHRISTENSEN 1953, LUNDGREN 1955). Dehydration also occurs as a result of a lack of water. Limited access to water can result from the contamination of drinking water by radiation, for example, or infectious agents or poison (during war), but is common also for persons lost in the mountains during winter or at sea. Extended sport competition can also give rise to marked dehydration; for example, there was a fluid loss of up to six to seven liters during an 85 km ski competition. The estimated sweating intensity is then up to 1 l/hr. Sweating intensity during summer is higher and in connection with long distance running (orientering 1.5 hr, marathon running) it goes up to 2 l/hr (unpublished results).

Many authors (for example PITTS et al. 1944, ADOLPH 1947, LADELL 1955) have stressed heavily the importance of fluid administration (which may include salt) and also shown that a greater dehydration than 1.5—2.0 % of the initial body weight implies a decrease of the work capacity. ADOLPH and associates' data on the reduced blood volume in dehydration has rendered obvious the explanation of the positive effects of fluid administration. Fluid and salt administration during and after extended work are probably not of crucial importance for the circulation. In preliminary studies of rehydration (including salt) during extended hard work the work capacity was remarkably better at maximal work after the work period than after exercise dehydration. A complete explanation of the positive effects of fluid administration during work cannot be given, however, until the negative effects of extended work are clarified. It is also certain that during extended work the fluid and salt problem is intimately connected with the problem of available energy depots.

BUSKIRK and BEETHAM (1960) thus summarize their observations from marathon running regarding pace and degree of dehydration:

Running speed (pace) was maintained essentially constant by each man in all races — there was no letdown in pace near the end of the race. In fact a sprint finish was frequently attempted. ... Thus well-conditioned men running in a cool environment seem to tolerate a three to seven per cent dehydration rather well.

The present study also has shown that the well trained subject tolerates dehydration better than the untrained, but on the basis of the data presented here it would be unrealistic to conclude that a dehydration of up to 7 per cent of the body weight is' rather well tolerated In the present study both trained and untrained subjects exhibited a gradual decrease in work capacity during increasing dehydration This was significant even at a very slight degree of dehydration (≈ 1 0 %) The greater the part played by metabolic heat as a stress factor, the more extreme was the decrease in work capacity Aerobic work capacity may be excluded as a factor essential to an explanation of the gradual decrease in physical work capacity occuring during dehydration The results may be interpreted to indicate that the explanation should be sought at the cellular level where changes occur during dehydration These changes are more marked after extended heavy work than after thermal dehydration

SUMMARY AND CONCLUSIONS

1 A determination of the oxygen uptake during work on a bicycle ergo meter exhausting the subject within two to eight minutes actually gives a measure of the individual s aerobic work capacity

2 The dye dilution technique and the acetylene method for the determination of cardiac output agree well at rest and during work up to maximal level and the reproducibility of the two methods was good

3 There was a minor rate of increase in cardiac output when oxygen uptake gradually approched maximum

4 There was a higher stroke volume during light exercise in upright work position than at rest but at a work load heavier than 40 % of the aerobic work capacity there was no further significant increase

5 A dehydration caused by a high environmental temperature (thermal dehydration) affected the plasma valume and the extracellular volume much more than if the same degree of dehydration was saused by severe exercise (exercise dehydration)

6 After six to ten hours skiing no significant reduction in red cell volume was found

7 At submaximal work in upright position there was after dehydration an exaggerated heart rate response which was more marked after exercise dehydration than after thermal dehydration In both situations the stroke volume was decreased as there were only minor changes in the cardiac output. Only after the thermal dehydration was the reduction in stroke volume correlated to the decrease in plasma volume

8 At maximal work in upright position there was after dehydration no change in either the central circulation or the aerobic work capacity compared with normalcy in spite of a body weight decrease of up to 5,5 %

9 The work time on the maximal work load was definitively reduced after dehydration and most markedly after the exercise dehydration As mechanical efficiency and aerobic work capacity were unchanged after dehydration the reduction in work time was accompanied by a lower peak blood lactic acid concentration

10 Reduced aerobic work capacity may be excluded as an essential explanation for the gradual decrease in physical work capacity during dehydration The results may be interpreted to indicate that the explanation should be sought at the cellular level

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EXTRA-ADRENAL CHROMAFFIN TISSUE OF THE RAT AND THE EFFECT OF CORTICAL HORMONES ON IT

BY

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HELSINKI 1964

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FROM THE DEPARTMENT OF ANATOMY UNIVERSITY OF HELSINKI
SILTAVUORENPENGER HELSINKI FINLAND

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PREFACE

The present investigation was carried out in 1961—1964 in the Department of Anatomy, University of Helsinki It was proposed by Professor Olavi Eränkö M D Ph D Head of the Department It is closely related to his research project (project N o A—1725 National Institute of Arthritis and Metabolic Diseases U S Public Health Service)

I wish to express my deep gratitude to Professor Eränkö for his interest encouragement and continued support of my endeavours in the course of the different stages of this study from the first pilot experiments to the preparation of the manuscript

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It is a pleasure to express my appreciation to Professor Antti Telkkä M D and Docent Mikko Niemi M D for their valuable advice

Further I thank my colleagues in the Department of Anatomy for their helpful attitude My thanks are due especially to Doctors Matti Härkönen Lic Med Boris Kock Lic Med Aulikki Kokko Lic Med Ulla Söderholm Lic Med and Kari Ojala Cand Med for many stimulating discussions and valuable instruction which were of great importance for the progress of this study

I am grateful to my sister Miss Anja Lempinen for drawing the diagrams

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Mr Frederick Clement has corrected the English text I thank him for his collaboration and interest in my work

Helsinki May 1964

Matti Lempinen

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REVIEW OF THE LITERATURE

CHROMAFFIN REACTION

Henle (1865) showed that the cells of the adrenal medulla possess a special affinity to the chrom salts and become brown when treated with them The cells which gave a positive *Henle's reaction* were called *chromophil* by Stilling (1890) *phaeochrome* by Poll (1906) and *chromaffin* by Kohn (1898) The latter term has been generally accepted and is universally used to-day

The nature of this *chromaffin reaction* has been a subject of numerous studies (*e g* Kohn 1902 1903 Ogata & Ogata 1917 Gerard *et al* 1930) After the work of Gérard *et al* (1930) it was for a long time accepted that the chromaffin reaction was due to the oxidation of adrenalin and related substances with the formation of quinones and tar like derivatives Coupland (1954 a) demonstrated that the compound produced by dichromate is different from that produced by iodate in that after formol-dichromate fixation it is chromium that is fixed by the cells of the adrenal medulla and suggested that a chromadrenochrome is produced On the other hand Eranko (1955) demonstrated that after fixation in a mixture of formaldehyde and potassium iodate iodine is fixed in an analogous way in the adrenal medulla presumably in the form of polymeric iodoadreno chrome derivatives

Numerous studies have shown that a fairly good correlation exists between catecholamine concentration determined by quantitative chemical methods and the intensity of the chromaffin reaction (*e g* Eranko 1955 a Hillarp & Hokfelt 1955 Coupland & Exley 1957 Eranko & Raisanen 1957 Coupland 1958 Eranko & Hopsu 1958) Eranko & Raisanen (1961) made a comparison of the photometrically measured intensity of the chromaffin reaction and the catecholamine concentration and a good correlation was found.

THE EXTRA ADRENAL CHROMAFFIN TISSUE

Mitsukuri (1882) Gottschau (1883) and Phaundler (1892) observed that the adrenal medulla of the rabbit embryo was not entirely enclosed by cortical tissue but that chromaffin cell cords came out freely from the gland being in a close continuity with the nearby sympathetic ganglia

Dostoiewsky (1886) observed that sympathetic ganglia themselves near the adrenals contained groups of cells with properties similar to those of the adrenal medulla He called those cells accessorische Nebennieren aus Marksubstanz"

Stilling (1890) described groups of cells giving a positive Henle s reaction in the midst of the abdominal sympathetic plexuses of the cat and the dog These cells were shown to be identical not only in their reaction to dichromate but also in morphological characteristics with the chromaffin cells of the adrenal medulla Restating his results (1898) he called these cells the chromophil cells and bodies of the sympathetic

Zuckerkandl (1901) first demonstrated a paired mass of chromaffin cells in the aortic plexus of the human embryo at about the level of the inferior mesenteric artery He called them Nebenorgane des Sympathicus and they are probably the homologues of the paraganglion aorticum lumbale" described by Kohn (1903) in his classic paper in which he gives a detailed description of the extra adrenal chromaffin tissue in man cat and rabbit

Many investigators have later confirmed the findings of Zuckerkandl and Kohn (*e g* Bonnamour & Pinatelle 190? Iwanoff 192? Wrete 1927 Iwanow 1930 Coupland 19?2) It is now generally accepted that in the human fetus and the newborn there is an abundance of chromaffin cell bodies in association with the abdominal sympathetic plexuses The main bodies are now usually referred to as the *Organ of Zuckerkandl*"

Similar formations have been reported in all mammals in which a careful study of the extra adrenal chromaffin tissue has been made *e g* Kohn (1903) rabbit cat Vincent (1910) dog, cat, rabbit mouse Pellegrini (1906) cat dog rabbit mouse Wislocki (1922) oppossum guinea pig squirrel monkey Goormaghtigh (19?8) mouse Coupland (19?) rabbit (1960) mouse guinea pig However the rat seems to be an exception and the existence of extra adrenal chromaffin tissue in this animal has been rather a question in dispute (see page 18)

A more detailed history of the early studies on the extra adrenal chromaffin tissue is given in several reviews *e g* Kohn (1902) Iwanow (1932) Hollinshead (1940) Watzka (1943) and van Campenhout (194?)

ORIGIN OF THE CHROMAFFIN TISSUE

When studying the development of the adrenals in the rabbit embryo Koelliker (1879) came to the conclusion that the adrenal medullary tissue was derived from the sympathetic elements which had become enclosed by the cortical tissue during development Similar observations were made by Mitsukuri (1882) and later these findings were confirmed in mammals by numerous investigators *e g* Wiesel (1901 1902) Inaba (1891) Fusari (1891) Soulié (1903) and others

Sympathetic origin of the chromaffin part of the adrenal gland was thus already accepted at the time of the discovery of the extra adrenal chromaffin

tissue (see above) Attention was then naturally directed to the embryology of the latter tissue Zuckerkandl (1901) supposed that the extra adrenal chromaffin bodies which he was the first to observe originated from the sympathetic elements Kohn (1903) showed the identity of the embryological origin of these two kinds of chromaffin cells He proposed that the chromaffin tissue formed a tissue "*sui generis* and that the paraganglia are genetically and anatomically related to the sympathetic nervous system The studies by Poll (1906) Vincent (1910) Keene & Hewer (1927) Wrete (1927) Iwanow (1930) Coupland (1952) and many others have supported Kohn s opinion The common embryological origin of the sympathetic and chromaffin tissue has been experimentally confirmed *e g* by the studies of van Campenhout (1930 b)) in amphibia and by Willier (1930) and Hammond & Yntema (1947) in birds

ORIGIN OF THE PRIMARY SYMPATHETIC CELLS

A theory of the *mesenchymal origin* of the sympathetic nervous system was proposed in 1847 by Remak Paterson (1890) assumed that the sympathetic tissue was derived from mesoderm in the region of the dorsal aorta More recently the experimental studies by Tello (1925) have given support to this view He concluded that the sympathetic elements differentiated *in situ* from mesodermal cells However the number of advocates supporting the mesodermal origin is very limited

Spinal ganglia as a source of the sympathetic tissue were suggested by Onodi (1886) The concept of the origin from the *neural crest* or spinal ganglia was described by His (1897) Neural crest origin of the sympathetic cells has later been supposed by various authors more recently *e g* van Campenhout (1931) and Creeven (1938)

Froriep (1907) supposed that the sympathetic cells originated from the *neural tube* and later the works *e g* by Kuntz (1909 1910 1911 1912) Goormaghtigh (1921) and Mihálik (1936 1940) have given support to this theory

The studies by Abell (1912) suggest that the primary source of the sympathetic nervous system is the neural tube and the neural crest and this view has been supported by the neurohistological studies by Uchida (1927) in mammals Numerous experimental studies made both on birds (usually chick embryos) and on amphibia have yielded results which indicate an origin from the neural crest or neural tube The latter site has been supported *e g* by Kuntz & Batson (1920) Uchida (1927) Jones (1937 1939 1941) and Brizzee & Kuntz (1950) while the works by Muller & Ingvar (1921 1923) van Campenhout (1930 b) Yntema & Hammond (1945) and Hammond & Yntema (1947) suggest that the sympathetic ganglia both in the paravertebral and prevertebral positions arise from the neural crest cells This is the most commonly accepted view to day A more detailed discussion about the origin of the sympathetic nervous system has been given by van Campenhout (1930 a) Mihalik (1940) and Yntema & Hammond (1947)

DIFFERENTIATION OF THE PRIMARY SYMPATHETIC CELLS

Assuming the neural crest as the site of origin of the sympathetic cells the following steps in the development of the sympathetic neurons have been suggested (Yntema & Hammond 1947)

1 Differentia the neural crest from its ectodermal rudiment 2 Migration of the cell neural crest 3 Differentiation of the sympathetic neurobla t n of the neuroblasts into ganglia

Part ural crest cells in the sympathetic anlage along the dorsolate l lominal aorta extend into the pre aortic region forms plexuses The primitive cells (sympathogonia Poll 1906 aracterized by small intensely basophilic nuclei about 4— & Hewer 1927 Coupland 1952 Ito 1959)

lls nervous cells of various kinds are formed ribed two kinds of cells arising from the common ll dark staining type which differentiated into nerve and a larger pale staining type which gave rise ll and to the cells in the side organs" of the

the sympathetic ganglion cells the Schwann s cells nd paraganglionic cells differentiate from primary cell subsequently differentiating into other than he accessory cell by him The accuracy of these irmed *e g* by Kuntz (1912) Wrete (1927) Iwanow upland (1952) Ito (1959) and others

Smitten (1958 1962 1963) and Prumak (1960) ll are derivatives of the glial elements of the sym ording to these studies chromaffin cells differentiate nding to the future Schwann s cells and satellite br cells from the primitive undifferentiated cells

FORMATION OF THE EXTRA ADRENAL CHROMAFFIN BODIES

ll within the primitive sympathetic plexuses are arranged in fashion forming little groups which gradually increase in According to Smitten (1963) a certain redistribution of the sympathetic ganglia takes place in early embryogenesis with a predominantly spongioblastic trend of development concentrate which chromaffin organs subsequently develop

Cells in these groups have moderately basophilic ovoid or round nuclei about in diameter and the amount of cytoplasm is greater than in the primary sympathetic cells (Coupland 1952 and others) These cells are phaeochromoblasts (Coupland) or chromaffinoblasts (Smitten) and they probably correspond to these large pale staining cells described by Zuckerkandl (see above)

The next phase in the cellular differentiation is characterized by a decrease in nuclear basophilia in the developing phaeochromoblasts Chromatin granules become more obvious the nucleus is round or ovoid and of about the same size as that in phaeochromoblasts There is also an increase in the amount of cytoplasm Morphologically these cells are identical with mature chromaffin cells (phaeochromocytes) except for the absence of chromaffinity from their cytoplasm Chromaffin reaction appears later (Coupland 1952)

According to Smitten (1963) there are some species dependent differences in the appearance of the chromaffinity in the cytoplasm of developing chromaffin cells In some animals the chromaffin reaction appears in little differentiated forms of cells still possessing cytological features of the spongioblasts (mouse and chick embryos) in other animals the reaction may be found only in more mature already specialized cells (rat rabbit embryos)

Together with the nuclear changes the cells become rearranged and they lose their original whorl like form These cell cords are associated with a network of capillaries and most of them are surrounded by a thin connective tissue capsule (Coupland 195?) These paraganglia (Kohn) or para aortic bodies (Coupland) extend in a cranio caudal direction and form groups of chromaffin cells associated with the sympathetic plexuses The final rearrangement of the cell masses somewhat varies from one species to another but usually a large medial encapsulated collection of chromaffin cells can be found representing the organ of Zuckerkandl The extra adrenal chromaffin bodies gradually increase in size when the fetus grows and they reach their maturity at birth or soon after (Coupland 1952 and others)

More detailed description of the development of the extra adrenal chromaffin tissue is given *e g* by Kohn (1903) Iwanoff (1925) Iwanow (1930) and Coupland (1952)

FORMATION OF THE ADRENAL MEDULLA

Independently of the differentiation of the extra adrenal chromaffin cells there is an extension of primary sympathetic cells from the ganglia adjacent to the medial side of the developing adrenals into the middle of the adrenocortical cell cords The differentiation of the pr mary sympathetic elements to phaeo chromoblasts and further to mature chromaffin cells takes place in the same way as that of the cells in the extra adrenal chromaffin bodies (Millier 19?6 Keene & Hewer 1927 Hammar 1934 Coupland 1952) However the differentiation of the chromaffin cells forming the adrenal medulla takes place later than that of those forming the extra adrenal chromaffin bodies and at the time when extra adrenal chromaffin tissue is almost mature primitive sympathetic cells and phaeochromoblasts are still dominant features in the adrenal medulla (Coupland 1952 and others)

CELL DIFFERENTIATION IN THE SYMPATHETIC GANGLIA

The early differentiation of the primitive c lls into sympathicoblasts takes place much later than that of future chromaffin cells At about the time when mature chromaffin cells are already detected in the developing para aortic bodies morphological changes begin in those cells differentiating into ganglionic cells (Coupland 195?) The first sign of differentiation is a decrease in nuclear basophilia According to Coupland (1952) Ito (1959) and many others the first stage sympathicoblasts are morphologically indistinguishable from phaeochromo blasts Sympathicoblasts nevertheless increase relatively quickly in size The nucleus is large and vesiculated with 1—2 nucleoli At this stage these sympa thicoblasts (probably corresponding to the neuroblasts of Smitten) are often

surrounded by small, dark, polynuclear cells with a narrow rim of cytoplasm. These small cells are glial cells (Smitten, 1963)

Further sign of differentiation of the sympathicoblasts is a gradual increasing in the size of the nuclei and the amount of cytoplasm, and the cells begin to show the multipolarity characteristic of the sympathetic ganglion cells (Ito 1959)

At the time of birth, sympathetic ganglia are very immature and there are sympathetic cells of all stages of differentiation and also undifferentiated primitive cells (Coupland, 1952 Keene & Hewer 1927) Also isolated chromaffin cells and small groups of chromaffin cells can be seen in the ganglia in most mammals. According to Smitten (1963) the differentiation of indifferent cells ends much sooner *e g* during 12—16 days of embryogenesis in rats 11—15 days in mice During these periods the undifferentiated cells take either the pongioblastic or neuroblastic direction of development and after that the nerve ganglion no longer contains any undifferentiated cells

GENERAL DISTRIBUTION AND APPEARANCE OF THE EXTRA ADRENAL CHROMAFFIN TISSUE

The topographical distribution of the extra adrenal chromaffin tissue has been described in detail both in human newborn (Zuckerkandl, 1901 Kohn, 1903 Iwanow 1930 1932 Wrete, 1927 Handschin, 1928 Coupland 1952) and in most of the common laboratory animals cat, dog, guinea pig, rabbit, mouse (Kohn 1903 Pellegrini, 1906 Wislocki, 1922 Vincent, 1910 Coupland, 1956 1960) However no previous description of the topography of that tissue has been made in the rat (see page 15

In all these mammals the extra adrenal chromaffin tissue appears either as encapsulated groups of chromaffin cells associated with the abdominal sympathetic plexuses or as isolated chromaffin cells or non-encapsulated cell masses within the abdominal and lumbar sympathetic ganglia Encapsulated cell groups predominate and as a rule the majority of the tissue is concentrated in the main bodies forming the organ of Zuckerkandl These bodies are either paired or connected by an isthmus, in human newborn at the level of the inferior mesenteric artery on both sides of the abdominal aorta Reconstructions of these bodies have been made *e g* by Wrete (1927) Iwanow (1930) Coupland (1952)

The topography of the chromaffin bodies is subject to species-dependent differences but usually they lie at the level of the renal veins between the superior and inferior mesenteric arteries and are either globular (guinea pig mouse) or elongated (dog cat, rabbit) Kohn (1903) Vincent (1910)

In general, the main para aortic body contains a few small groups of ganglion cells but chromaffin cells predominate Mitotic figures are usually present at the time of birth. Collagen fibres run in the body which is richly supplied with blood capillaries (see Coupland, 1952 1960 and others) The histology of the smaller chromaffin bodies is similar to that of the main body

The relative amounts of extra adrenal and intra adrenal chromaffin tissue have been estimated, but the reports are variable Kahn (1912) calculated that the main chromaffin body of the dog represented only about one fourth to one tenth of the volume of the adrenal medullary tissue Pearlman & Vincent (1919)

reported that the total mass of the extra adrenal chromaffin tissue may exceed that contained in the adrenal glands However no quantitative measurements of the total volume of the extra adrenal chromaffin tissue have been made

INNERVATION OF THE EXTRA ADRENAL CHROMAFFIN TISSUE

Jacobi (1892) studied the adrenals of the rabbit experimentally by cutting the splanchnic nerves and found that most of the nerve fibres to the rabbit adrenals were preganglionic Dreyer (1899) demonstrated that stimulation of the nerves to the adrenal glands leads to an augmented output of adrenalin into the blood stream Elliot (1913) suggested that the chromaffin cells in the adrenal medulla are directly innervated from the primary preganglionic nerves without the intervention of ganglion cells The studies *e g* by Hoshi (1927) Hollinshead (1936) and Swinyard (1937) have confirmed these findings (for further references see *e g* Alpert (1931) Feldberg *et al* (1934) showed that acetylcholine is released in the adrenal medulla after splanchnic nerve stimulation

Many investigators *e g* Zuckerkandl (1901) Wetschtomow (1910) Jachontow (1913) and Iwanow (1930) had failed to find nerves in the extra adrenal chromaffin bodies Ciardi Dupré (1936) demonstrated some ganglion cells and nerve fibres in the para aortic bodies but according to him this was not a constant feature However Smirnow (1890) Kohn (1903) and Dowgjallo (1926) managed to demonstrate a network of fibres interpreted as nerve fibres in the paraganglia after methylene blue staining Pines (1924) Handschin (1928) Kofmann (1937) and Bakay (1938) reported that there is a contact between the paraganglionic chromaffin cells and sympathetic nerve fibres The studies by Hollinshead (1937) suggest that at least the main para aortic chromaffin bodies have the same type of preganglionic innervation as the chromaffin cells of the adrenal medulla

FUNCTION AND IMPORTANCE OF THE EXTRA ADRENAL CHROMAFFIN TISSUE

The pressor effect of extracts of the adrenal medulla was first observed by Oliver & Schafer (1895) Biedl & Wiesel (1902) reported similar effects on the blood pressure with extracts from the organ of Zuckerkandl Later these observations have been confirmed *e g* by Vincent (1910) Kahn (1912) Fulk & MacLeod (1916)

Takamine (1901) isolated the active principle of the adrenal medulla and gave it the name adrenalin

That the adrenal medulla contains noradrenalin in addition to adrenalin has recently been proved by numerous investigators (for references see Euler 1956)

While adrenalin is the main catecholamine in the adrenal medulla the pressor effect of the extra adrenal chromaffin tissue has been reported to be almost entirely due to noradrenalin (Shepherd & West 1952 Coupland 1953)

The works of Gaskell (1920) pointed out that in the lowest vertebrates the function of the sympathetic nervous system is largely taken over by the chromaffin system It has been mentioned previously in the present paper that the extra adrenal chromaffin tissue is mature at the time of birth and that the

adrenal medulla and sympathetic nervous system are still at a more primitive stage of development at that time (Coupland 1952 and others)

Hunter MacGregor *et al* (1953) and Niemineva & Pekkarinen (1953) observed that the amounts of pressor amines extracted from the organ of Zuckerkandl in human fetuses exceeded those found in the adrenal medulla of the same fetus At the time of birth the main para aortic bodies contain more noradrenalin than the adrenal medullary tissue and the extra adrenal chromaffin tissue has been assumed the main source of pressor amines in the human infant at least during the first year of life (Hunter MacGregor *et al* 1952) All these studies suggest that the extra adrenal chromaffin tissue functions as a real endocrine organ concerned with the humoral control of vasomotor tone at a time when the adrenal medulla and the sympathetic nervous system are still at a more primitive stage of development (Danisch 1926 Coupland 1953)

POSTNATAL FATE OF THE EXTRA ADRENAL CHROMAFFIN TISSUE

Wiesel (1902) proposed that the development of the side organs of the sympathetic has been completed during the embryonal stage while that of the adrenal medulla reaches its full maturity later during the postnatal life He suggested that the extra adrenal chromaffin tissue persists in man up to the time when the adrenal medulla has reached its maturity

Bonnamour & Pinatelle (1902) observed in a 5—6 year old infant extra adrenal chromaffin bodies not very much different from those of the newborn Zuckerkadl (1901 1912) found degenerative changes in those organs in a 2 1/2 year old child but in another child 5 years old [illegible] res were well developed

Scheel (1908) studied the coeliac p [illegible] ganglia of the lumbar sympathetic chain in human infants and he [illegible] chromaffin cells in these ganglia were fully degenerated at the

According to Handschin (19[illegible]) [illegible] in of Zuckerkandl begins already at the end of the [illegible] erty but she [illegible] noticed groups of faintly positi[illegible] in in a 20 year old human being

Iwanow (1930 1932) div[illegible] adrenal [illegible] affin tissue into two main [illegible] the birth 1—1 1/2 years the main p[illegible] was [illegible] by him paraganglion aorticum [illegible] The phase was characterized not or [illegible] als degenerative changes with a [illegible] Zuckerkandl which was now [illegible] decidens

The studies of Iwanow have [illegible] in man According to his [illegible] increase in size up to the age [illegible] changes supervene in all bodies [illegible] discrete para aortic bodies exist [illegible] mesenteric arteries whilst only sma[illegible] are associated with the lower pre[illegible]

Kohn (1903) studied the extra adrenal chromaffin tissue in the rabbit cat and man He came to the conclusion that degeneration in the human paraganglia takes place already during childhood However in the other two mammals the chromaffin bodies are permanent organs The studies by Vincent (1910) confirmed this opinion He has described the main abdominal paraganglia in the adult dog cat and rabbit However Pellegrini (1906) found degenerative changes in the extra adrenal chromaffin bodies of the cat dog rabbit and mouse Recently Vanov & Vogt (1963) observed clusters of chromaffin cells in the inferior mesenteric ganglion of the rabbit

According to Goormaghtigh (1928) the main para aortic body is present in the newborn mouse but not in the adult

Coupland (1956 1960) studied the postnatal fate of the para aortic bodies of the rat rabbit guinea pig and mouse According to him a well defined healthy looking body can be found in the adult rabbit and the guinea pig In the mouse as in man the main body loses its compact form and some of the pre-aortic chromaffin cells undergo degeneration and disappear

Coupland failed to find any extra adrenal chromaffin tissue in the rat (see page 18)

DEGENERATION OF THE EXTRA ADRENAL CHROMAFFIN CELLS

Zuckerkandl (1912) described hyaline degeneration in the main paraganglia in man According to Pellegrini (1906) the degenerative changes were characterized by dislocation of the organ of Zuckerkandl decrease in chromaffinity and increase in the connective tissue stroma Degenerative changes described by Iwanow (1930 1932) included hyperaemia and lymphoid infiltration decrease in chromaffinity vacuolization and hyalisation of the cytoplasm nuclear pyknosis and irregularity were also observed The cellular degeneration was associated with gradual diminution in the size of the organ of Zuckerkandl The final stage was fibrosis

Handschin (1928) also described a disappearance of chromaffin cells in the main para aortic body of the human infant the connective tissue increased while the total mass of the paraganglionic tissue decreased Small groups of faintly staining chromaffin cells were found in adult man among the fibrous and adipose tissue in the region formerly occupied by the organ of Zuckerkandl

Goormaghtigh (1928) proposed that the disappearance of the chromaffin bodies in the mouse was the result of migration of the cells from the pre aortic region

According to the observations by Coupland (1954 b 1956 1960) the postnatal changes in the main para aortic bodies of man rabbit guinea pig and mouse are passive and result from mechanical forces produced by the differential growth of sympathetic nerve fibres and contiguous chromaffin elements Cellular infiltration and wide spread degeneration of the chromaffin bodies were not observed by Coupland According to him the increase in connective tissue is not *per se* a sign of degeneration

CHROMAFFIN AND RELATED CELL GROUPS IN OTHER PARTS OF MAMMALIAN ORGANISM

Groups of cells giving a positive chromaffin reaction have been found in various organs e g in the kidney heart ovary etc (for references see Iwanow 1930 1932 Watzka 1943) The real nature of these cell groups is obscure

Kohn (1902) proposed the name "paraganglia to all those collections of cells which were developmentally and topographically related to the sympathetic nervous system and gave a positive Henle s reaction Kohn included the carotid body in his paraganglia It is well known that cells can be found in the carotic body of several mammals e g man cat rabbit and pig which give a positive chromaffin reaction However according to Castro (1926 1928) these cells are not true chromaffin cells either developmentally or functionally Nevertheless it has been shown by numerous other authors e g Watzka (1934) Boyd (1937) and Winiwarter (1939) that real chromaffin cells can be found in the carotid body although the cells of this organ as a whole do not show this reaction

It is not possible to penetrate in the present paper into the voluminous literature on the carotid body (for more detailed discussion and references see for ex Hollinshead 1940 Watzka 1943 and van Campenhout (1946) It suffices to say that more recently the works of Rahn (1961) have given further support to the view that chromaffin cells found in the carotid body are identical with those forming the abdominal chromaffin bodies

It is now generally accepted that the so called cardio aortic bodies usually referred to as paraganglia in the supracardial and lower cervical regions of mammals are not identical with the abdominal para aortic bodies The same can be said about the so called coccygeal body Cells in those bodies do not give chromaffin reaction and the name "paraganglia in this connection is misleading (Hollinshead 1940 van Campenhout 1946)

Goormaghtigh (1936) described a non chromaffin paraganglia in the adult mouse which he called vagal paraganglia The real nature of these non chromaffin cell bodies is obscure but it is very likely that they are not related to the real paraganglia i e the abdominal chromaffin bodies either developmentally or functionally (see van Campenhout 1946)

CATECHOLAMINES OF THE CHROMAFFIN CELLS

It is now generally accepted that there are in the adrenal medulla two different types of chromaffin cells one type containing adrenalin and the other noradrenalin Various methods have been used in order to distinguish these two cell types In demonstrating these noradrenalin cells two specific methods have been generally used i e the formalin induced fluorescence method (Eränko 1951 1955b 1958) and the iodate reaction (Hillarp & Hokfelt 1953 1955) These two methods selectively demonstrate the same types of medullary cells i e those containing noradrenalin (for further references see e g Hopsu, 1960)

The pressor effect of extracts from the extra adrenal chromaffin tissue has been reported to be almost entirely due to noradrenalin (Shepherd & West 1952 Coupland 1953 and others) However the chromaffin reaction in its various

modifications equally demonstrating adrenalin and noradrenalin has been almost solely used for the study of this tissue

Muratori & Battaglia (1960 a) have proved a positive iodate reaction in the main para aortic body of the cat and this appears to be the only report on histochemical demonstration of noradrenalin in the extra adrenal chromaffin tissue

It has been discussed in the present paper that cells can be found giving a positive chromaffin reaction in the carotid body of some mammals Muratori & Battaglia (1960 b) demonstrated cells in the carotid body of the pig which gave a positive iodate reaction and fluoresced brilliantly in ultra violet light Rahn (1961) has in his detailed study observed a distinct correlation between the intensity of the chromaffin reaction and the amount of iodate positive and fluorescent cells and the amoun of pharmacologically demonstrated nor adrenalin in the carotid body of the pig and the calf

THE POSSIBLE INFLUENCE OF THE ADRENAL CORTEX ON THE CHROMAFFIN CELLS

Kuntz (1912) suggested that the differentiation of the primary sympathetic cells to the chromaffin cells was stimulated by hormones secreted from the adrenal cortex However until now the possible functional relationship between the adrenal cortex and differentiation of chromaffin cells has remained largely a matter of speculation although the possible role of the adrenal cortex in the later differentiation of the chromaffin cells i e in the formation of cells secreting adrenalin has been discussed by various authors

That noradrenalin is a primary amine and serves as a precursor of adrenalin and is converted into the latter by methylation has been shown by the investigations of Blaschko (1942) Bulbring (1949) and others

Shepherd & West (1951) proposed that there was a direct relationship between the cortex medulla ratio and the percentage of adrenalin in extracts of mammalian adrenal glands

It has been mentioned in the present survey that the pressor effect of the extracts of the extra adrenal chromaffin tissue has been said to be entirely due to noradrenalin and only traces of adrenalin have been demonstrated from this tissue in older animals (Shepherd & West 1953)

Coupland (1953) studied the chromaffin tissue of man and the rabbit frog and dogfish In the dogfish whose chromaffin tissue is completely separate from the adrenocortical material the pressor effect of the tissue was entirely due to noradrenalin In the frog the noradrenalin accounted for about one half of the total pressor activity In man the intra adrenal tissue contained a mixture of adrenalin and noradrenalin whilst the pressor effect of the extra adrenal chromaffin tissue was entirely due to noradrenalin In the rabbit the intra adrenal chromaffin tissue is continuous with the extra adrenal chromaffin cells and while there was practically only adrenalin in the adrenal medulla of this spesies the chromaffin tissue outside the glands was reported to contain predom inantly noradrenalin

On the bassis of these findings Coupland concluded that the adrenal cortex is in some way associated with the methylation of noradrenalin and that if

there is a special medullotrophic factor secreted by the adrenal cortex this may reach a sufficient blood level in older animals so as to affect the extra adrenal chromaffin cells which would explain that adrenalin can be found even in the extra adrenal chromaffin tissue of older animals

EXTRA ADRENAL CHROMAFFIN TISSUE IN THE RAT

Although it is generally accepted that there is chromaffin tissue in association with the sympathetic plexuses in all other mammals studied the rat seems to be an exception

This tissue has been very little studied in the rat Vincent (1910) failed to find chromaffin tissue outside the adrenals in the rat he did not mention how old the animals examined were In the same paper he writes that Kohn had found an abdominal paraganglion in a rat and had sent an illustrative sketch to him

Wislocki (1922) studied with the naked eye after dichromate fixation the retroperitoneal region of the adult rat but he did not find extra adrenal chromaffin bodies

Wiswell (1952) studied with the unaided eye what he thought to be abdominal paraganglia in the rat and measured them in situ The spindle shaped nearly white glistering masses he described reached their maximum length in the age of 6 months and even in the adult their size was as big as 7 mm x 1—2 mm However since he did not use the chromaffin reaction to demonstrate these structures it is likely that his demonstration refers to some other tissue

In a microscopic study Coupland (1960) failed to find abdominal chromaffin bodies in a 1 day old rat but in a 7 day old specimen he found a small collection of chromaffin cells in the retroperitoneal tissue outside the adrenals He concluded that in the abdomen of the rat the chromaffin tissue is virtually confined to the adrenal glands

Pankratz (1931) has studied the development of the adrenal glands in the rat Formation of the medullary tissue takes place in the same way as in other species On about the 16th day of pregnancy cells begin to migrate from the neighbouring sympathetic ganglia into the adreno cortical cell masses Migration of those cells continues till birth Pankratz studied only the ganglia located near the adrenals He did not mention when the chromaffin reaction becomes positive in the medullary tissue

Smitten (1963) reported a positive chromaffin reaction in the adrenal medullary cells of the rat on the 19th day of embryogenesis

The adrenal medulla of the rat is very ill defined at birth and the chromaffin reaction in the medullary cells is weak Also the medulla and cortex have not yet become separated from each other (Dewitzky 1912 Jackson 1919 Franko & Raisanen 1957)

MATERIAL

White rats of *Sprague Dawley* strain were used as experimental animals. Their age varied from 1—2 hours after birth to adult. Both males and females were used. Fetuses from 15 days to full term pregnancy were studied for the appearance and localisation of the chromaffin tissue during the prenatal period. The age of fetuses was determined from the time of copulation, for which the females and males were put into the same cage for 2—4 hours. The females were either in the late stage I or in the stage II of Estrus cycle determined from vaginal smears.

Adult animals were fed on a standard diet and allowed to drink tap water. Both normal and experimental litters were freely taken care of by the mothers up to the time of puberty.

The animals were killed by decapitation with scissors. Fetuses were taken out through abdominal section under aether anaesthesia.

The retroperitoneal region including both kidneys and adrenals were removed as a block immediately after killing. Blood from the vessels was removed by gentle compression with blotting paper before immersion of the blocks into fixatives. In older animals from 20 days after birth both kidneys and the vertebral column were carefully cut out from the retroperitoneal block. For demonstration of chromaffin tissue in other parts of the sympathetic nervous system a tissue block was taken containing cervical ganglia and vessels and another including the ganglia of the thoracic sympathetic chain.

METHODS

THE CHROMAFFIN REACTION

The retroperitoneal tissue block was immersed in a mixture of 8 parts of 5 per cent potassium dichromate and 2 parts of 3.5 per cent neutral formaldehyde. After 24 hours the mixture was replaced with 5 per cent potassium dichromate for another 24 hours. The block was then washed in running water and immersed in 3.5 per cent neutral fomaldehyde for 5—10 days. The formalin solution was changed several times during the first days until it stayed colourless.

Serial sections were then cut with a freezing microtome at 40—90 micra and these were mounted on slides with glycerol jelly.

THE SCHMORL'S REACTION

In order better to distinguish the isolated faintly chromaffin cells in the *sympathetic ganglia Schmorl's ferro ferricyanide technique* was used (see Pearse 1961).

For this procedure the tissueblock from one animal of each series was cut with a freezing microtome at 40 micra after dichromate fixation (see above). Serial sections were collected on slides fixed in absolute alcohol for 1—2 minutes dried by gentle compression with blotting paper and carried through alcohol series back to distilled water. Staining was carried out in a solution of 3 parts of 1 per cent ferric chloride and 1 part of freshly made 1 per cent potassium ferricyanide for 5—10 minutes. The mixture was prepared immediately before use and used only for one staining. The sections were then dehydrated in alcohol carried through xylene and mounted in canada balsam.

HISTOLOGICAL STAINING METHODS

One or more animals from each series were used for these studies Bodian's number 2 fixing fluid (90 cc of 80 per cent alcohol, 5 cc of glacial acetic acid and 5 cc of neutral formaldehyde see § 1817 in Romeis 1948) was used as a fixative into which the retroperitoneal tissue block was immersed after killing The fixing time varied from 7—15 days depending on the size of the block Thereafter the post natal specimens were decalcified in a solution of 5 per cent thrichloracetic acid for 3—7 days depending on the age of the animal

The block was then dehydrated in alcohol series carried through butanol to paraffin in a tissue processing machine embedded in paraffin wax and sectioned serially at 5 and 10 micra

The following staining techniques were used

Haematoxylin eosin (see § 659 in Romeis 1948)

Van Gieson staining (§ 708 in Romeis 1948)

Haematoxylin orcein picroindigocarmin This method was used for indentification of the connective tissue elements After staining the nuclei with *Weigert's Haematoxylin* (§ 677 in Romeis 1948) the specimens were stained with *Unna's Orcein method* (§ 1556 in Romeis 1948) and finally with *Picroindigocarmin* (§ 718 in Romeis 1948)

Protargol impregnation by Bodian In principle the staining procedure was carried out after the original Bodian technique (Bodian 1937 § 1816 in Romeis 1948) as modified by Humphrey (1961) Protargol S (WINTHROP) was used instead of the old Protargol. Protargol solution (1 per cent) was prepared by sprinkling the powder on the top of the solution Copper foil in thin sheets was used and the copper was usually placed on the bottom of the staining dish. As a reducing solution hydroquinone (1 mg) and formalin (5 cc) in 100 cc distilled water was most succesfully used Reducing time varied from 2 to 10 minutes Staining in 1 per cent of oxalic acid was carefully checked with the microscope avoiding overstaining

QUANTITATIVE METHODS

VOLUME OF THE EXTRA ADRENAL CHROMAFFIN TISSUE

Specimens thoroughly fixed in dichromate and formalin (see above) were cut serially at 90 micra from the upper pole of adrenals to the lower end of the kidneys using a freezing microtome In order to minimize the error in the section thickness the same microtome was used for cutting all specimens Sections were rinsed in distilled water and mounted on slides with glycerol jelly

Every section was then carefully studied under the microscope the magnified image (120 x) was projected onto a horizontal table and the chromaffin cell islets were drawn on paper All drawings were made only by the present author

The drawings were then connected with lines and the area measured with a planimeter (see Eränko 1955 c)

Only those cell groups which were almost exlusively composed of chromaffin cells and thus homogeneously yellow were drawn so that e g sympathetic ganglia

with only some isolated chromaffin cells were not included In order to get the total volume of the extra adrenal chromaffin tissue in terms of mm³ the sum of the measured projected areas (mm²) was divided by the square of the linear magnification used (x 120) and multiplied by the section thickness (0 09 mm)

VOLUME OF THE ADRENAL MEDULLA

Adrenals were cut together with the tissue block at 90 micra The lines of the medullary tissue were drawn on paper and the volume was calculated as described above

STATISTICAL ANALYSIS

In the statistical analysis the testing was based on ranking using the Wilcoxon test (see Snedecor 1957)

PART I

STUDIES ON NORMAL MATERIAL

LATE FETAL DEVELOPMENT AND POSTNATAL FATE OF EXTRA ADRENAL CHROMAFFIN TISSUE IN THE RAT

MATERIAL AND METHODS

Altogether 45 fetuses and 160 post natal rats were studied The number and ages of the fetuses were as follows 10 of them were killed 15 days after copulation 6 after 16 days 7 after 17 days 7 after 18 days 5 after 19 days 5 after 20 days and 5 21 days after copulation

Serial frozen sections were made after dichromate formol fixation of 36 fetuses The others were used for histological studies using the van Gieson staining Fetuses of each age group were litter mates

The retroperitoneal tissue block of 120 young rats was serially sectioned after dichromate formol fixation Of these rats 100 were between o and 15 days of age (2—10 animals on every successive day)

From this material section series from 75 rats were used for quantitative measurements of the volume of extra adrenal chromaffin tissue From the section series prepared of the other rats either some sections were lost or there were variations in the section thickness so that they were not suitable for quantitative studies

14 dichromate formol fixed animals (one or two on every successive day during the first week after birth) were also studied after the Schmorl s ferro ferricyanide staining

One or more rats on every successive day during the first two post natal week were taken for histological studies using van Gieson Hematoxylin eosin Hematoxylin orcein picroindigocarmin and Bodian silver impregnation methods

Cervical and thoracic sympathetic ganglia were studied from 14 dichromate formol fixed rats (from newborn to 5 day old) One animal in each age group was examined also after the Schmorl s staining

RESULTS

MICROSCOPIC STUDIES

EXTRA ADRENAL CHROMAFFIN BODIES

In 16—17 day old fetuses small encapsulated groups composed mainly of two types of cells were seen on the lateral aspects of the developing pre aortic plexuses Nuclei of those cells were round or ovoid and about of the same size (6—8 micra in diameter) The nucleus of the first type of the cells was moderately basophilic containing 2—3 nucleoli These cells were probably phaeochromo blasts In the other cells the nuclei were less basophilic chromatin granules were more visible and the cytoplasm of the cells was larger than that in phaeochromoblasts These cells probably corresponded to mature chromaffin cells Occasional cells with smaller and darker nuclei and with scanty cytoplasm could be found in these bodies but their number was small when compared with those seen in the adjacent sympathetic ganglia The names extra adrenal chromaffin bodies or para aortic bodies were used to describe these encapsu lated structures

Topographically 5 different chromaffin bodies were observed The paired upper bodies were closely associated with the primitive sympathetic ganglia adjacent to the lower medial pole of the developing adrenal glands (Fig 1) and the lower encapsulated pair of bodies lay medial to the developing metanephros at about the

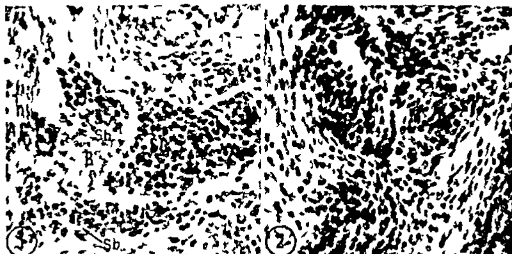


Fig 1 — 17 day old rat fetus Transverse section showing the developing suprarenal ganglion (SC) and a small para aortic body in the middle of it (B) Cells in the body are dominantly mature chromaffin cells or phaeochromoblasts In the ganglion young sympathicoblasts can be seen (Sb) but primitive cells with darker and smaller nuclei dominate Adrenal cortical tissue (AC) is seen lateral to the ganglion van Gieson x 200

Fig 2 — 17 day old fetus Transverse section at about the level of the origin of the superior mesenteric artery Small para aortic body (B) seen closely associating with the future coeliac ganglion (SG) Whorl like arrangement of the cells in the body can be noticed A small part of developing metanephros is seen lateral to the body (M) van Gieson x 200

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level of the origin of the superior mesenteric artery in the immediate vicinity of the future coeliac ganglion (Fig 2)

Below these two paired bodies a large encapsulated collection of phaeochromoblasts and chromaffin cells were seen in front of the aorta and the vena cava just caudally to the developing superior mesenteric ganglion (Fig 3) At this stage the upper end of the body was closely associated with the developing sympathetic ganglion (Fig 6) This main para aortic body corresponding to what is often called the organ of Zuckerkandl was composed of two longitudinally running parallel cell cords which were closely connected together forming a quite uniform structure The cells in this main body as also in the smaller para aortic bodies were arranged in a peculiar whorl like fashion (Fig 4)

Developmental changes soon took place in the main para aortic body When the fetus grew the body gradually elongated and thus became larger Already in a 17 day old fetus the paired cell cords

has fused together (Fig. 5). Fusion continued with age and finally the body formed a uniform encapsulated structure which to some extent resembled a downward narrowing funnel compressed into the corner between the aorta and the vena cava (Fig. 7 and 8). The upper extremity, very often paired, formed little horns and the body lay so that the horns were anterior to the left renal vein. Very often the right horn embraced the overcrossing vein. The whorl like arrangement of the cells was lost, these being scattered diffusely around the blood capillaries which gradually increased in number.

Simultaneously with the morphological changes the cells in the body underwent further differentiation and at the time of birth most of them were mature chromaffin cells and only occasional phaeochromoblasts and more primitive cells could be found in the body.

Also the smaller para-aortic bodies increased in size as the fetus grew. Cellular differentiation took place in the same way as in the main body and at birth also these small bodies were composed predominantly of mature chromaffin cells.

A selected series of sections illustrating the topography of the main para-aortic body and the lower lateral pair of smaller chromaffin bodies and the abdominal sympathetic ganglia at birth is presented in Figs. 9—20.

At birth the cells in the main para-aortic body were predominantly healthy looking chromaffin cells arranged around blood capillaries (Fig. 21). Their nuclei were round or ovoid, about 6—8 micra in diameter. Cytoplasmic vacuolization was rare. Mitotic figures were regularly present (with an average frequency of 5—10 in a 10 micra section). The body contained a scanty reticular type of connective tissue stroma.

Soon after birth the body underwent marked histological changes. As the animal grew the body gradually elongated becoming narrower and more flattened. However it usually kept its compact structure during the first two postnatal weeks, sometimes longer. Collagen fibres gradually increased in the body and chromaffin cells were replaced by continuously increasing sympathetic ganglion cells and fibres. The chromaffin body finally disintegrated usually during the third or fourth postnatal week. Thereafter only occasional chromaffin cells were found in this region.

When the body still formed a uniform structure wide spread cellular degeneration was noticed in the chromaffin cells. In 3 day

old rats (Fig 22) the main body was still composed of healthy chromaffin cells but soon (in 5—6 day old animals Fig 23) nuclear irregularity and pyknosis were prominent features and cytoplasmic vacuolization was commonly seen in the chromaffin cells However numerous healthy chromaffin cells could be found throughout the body Degenerative changes were marked and wide spread at the end of the first postnatal week (Fig 24) At the age of 10 to 15 days (Figs 25 and 26) pyknotic nuclei supervened and only some morphologically healthy chromaffin cells were left among the degenerated cells and gradually increased connective tissue stroma It was more difficult to follow the final fate of the isolated chromaffin cells after the disintegration of the body However it seemed that these scattered cells did not survive and their number gradually decreased with age

The postnatal fate of the smaller para aortic bodies was essentially similar to that of the main body The cells underwent gradual degeneration and finally disappeared Collagen fibres increased in the bodies and in older animals it was quite impossible even to recognize these former bodies with certainty (Figs 27 and 28)

Fig 3— 16-day old fetus Transverse section through the paired main para aortic body (B) showing its relation to the great abdominal vessels (Ao) aorta (Vc) vena cava van Gieson x 150

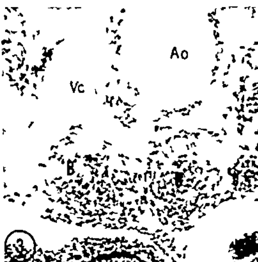
Fig 4— 16-day old fetus Transverse section through the middle of the main para aortic body The peculiar whorl like arrangement of the cells in these paired cell cords can be seen van Gieson x 300

Fig 5— 17-day old fetus Transverse section through the middle of the main para aortic body The paired cell cords begin to fuse together Most of the cells are morphologically mature chromaffin cells van Gieson x 300

Fig 6— Transverse section through the upper ends (B) of the same body as in fig 5 showing the close continuity of the body with the developing sympathetic ganglion (SG) Majority of the cells in the sympathetic ganglion are primitive sympathetic cells with dark and small nuclei van Gieson x 300

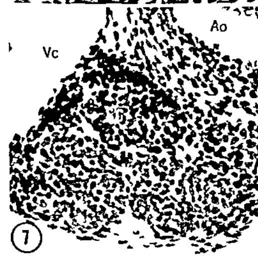
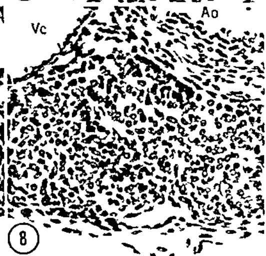
Fig 7— 19 day old fetus Transverse section through the main body immediately below the level of the left renal vein which crosses the upper end of the body The body has begun to assume its final form. A group of developing sympathetic cells (SG) primary cells and young sympathicoblasts can be seen in the corner of the aorta and vena cava van Gieson x 300

Fig 8 — 21 day old fetus Transverse section through the middle of the main para aortic body The body has taken its final form The cells predomiantly chromaffin cells have rearranged and lost their original whorl like arrangement. van Gieson x 300


Ao
Vc
B
3

4

Vc

Ao
Vc
SE
B

Ao
Vc
7

Ao
Vc
8

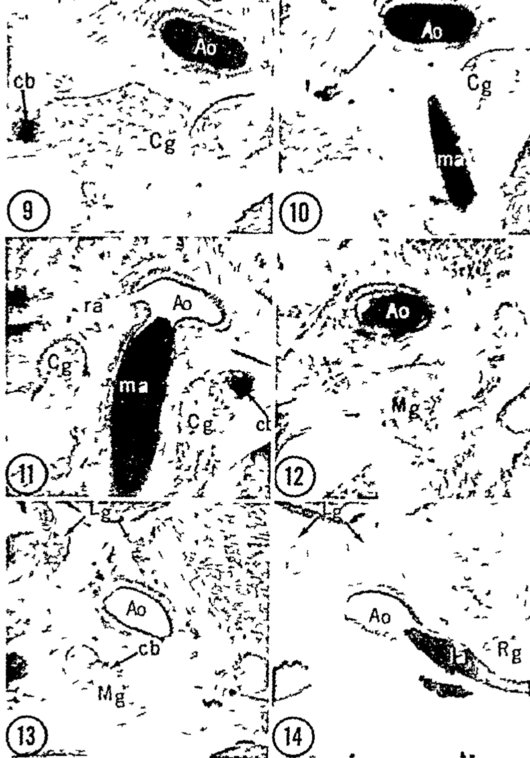
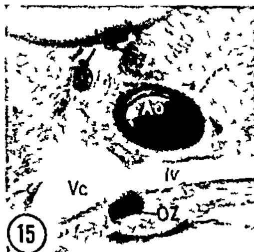
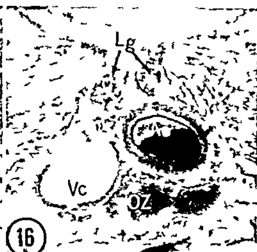
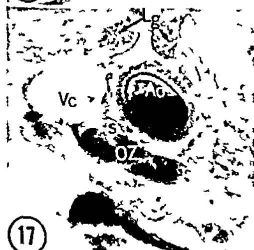
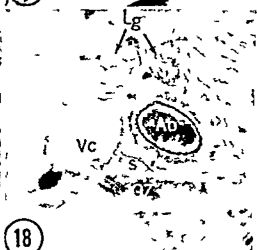


Fig 9–2[illegible] [illegible] transverse sections from the origin of [illegible] [illegible] the lower end of the main para-aortic body sh[illegible] [illegible] of the [illegible] and lumbar sympathetic ganglia and extra [illegible] third section has been taken [illegible] 30 micra [illegible]

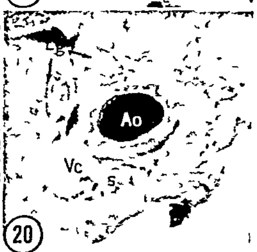












Ao = aorta Vc = vena cava ma = superior mesenteric artery ra = right renal artery la = left renal artery lv = left renal vein Cg = coeliac ganglion Mg = superior mesenteric ganglion Rg = renal ganglion Lg = lumbar ganglia

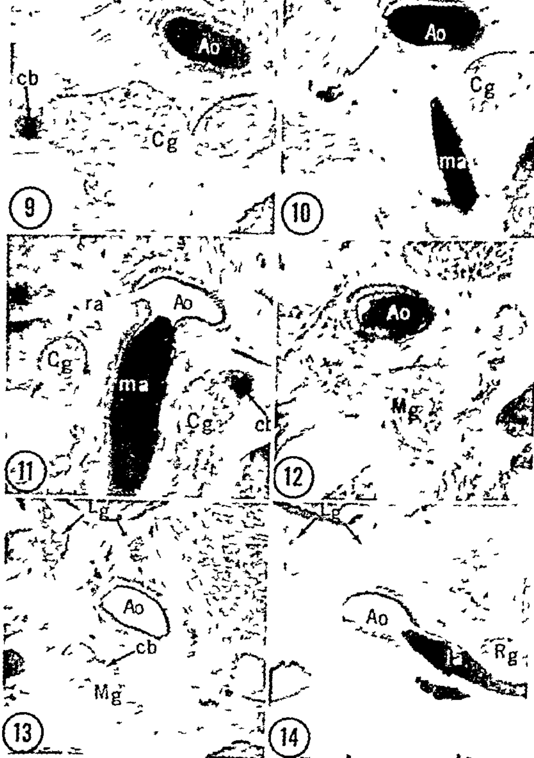


Fig 9–20 [illegible] A [illegible] series of transverse sections from the origin of the [illegible] artery to the lower end of the main para-aortic body, show[ing] [illegible] abdominal and lumbar sympathetic ganglia and extra [illegible] Every second or third section has been taken [illegible] fixed frozen sections at 90 micra [illegible]



Fig 27 — 6 day old rat A small para aortic body (PAB) closely associating with the coeliac ganglion (CG) Majority of the cells in the body still healthy looking chromaffin cells (At this stage the smaller para aortic bodies are usually completely non chromaffin) Sympathetic ganglion is morphologically mature, van Gieson x 250

Fig 28 — 8 day old rat Same para aortic body as in fig 27 (PAB) The body has lost its continuity with the coeliac ganglion (CG) Nuclear irregularity and pyknosis are predominant features however some healthy chromaffin cells can be seen among the degenerating chromaffin tissue van Gieson x 250

Transverse sections through the middle part of the main para aortic body van Gieson x 300

Fig 21 — Newborn rat Majority of the cells are mature chromaffin cells Mitotic figures are present (m) Developing sympathetic ganglion cells (Sb) are seen in the dorsal part of the body

Fig 22 — 3 day old rat The body is more flattened The cells are still healthy looking chromaffin cells Connective tissue stroma is scanty

Fig 23 — 6-day old rat Nuclear irregularity and pyknosis is observed in the chromaffin cells of the body Numerous healthy chromaffin cells still to be seen The amount of connective tissue is increased Mature ganglion cells (Gc) are seen in the dorsal aspect of the body

Fig 24 — 8 day old rat Nuclear irregularity and pyknosis are more marked than earlier Healthy chromaffin cells can be seen in the body but their number has diminished The amount of connective tissue has increased Sympathetic ganglion cells (Gc) are seen as in earlier stage

Fig 25 — 10-day old rat The body shows signs of disintegration though has still kept its compact structure Healthy chromaffin cells (Cc) are few Degenerating chromaffin tissue is predominant

Fig 26 — 15 day old rat Degenerating chromaffin tissue is the predominant feature in the body which has partly lost its compact structure Occasional chromaffin cells (Cc) can be seen seen among the sympathetic ganglion cells (Gc) and degenerated chromaffin tissue

In the 16th day after copulation the para aortic bodies were first seen to give a faintly positive chromaffin reaction (Fig 29) The intensity was similar both in the main para aortic body and in the smaller bodies The intensity gradually increased with age (Fig 30) receiving its maximum at about the time of birth (Fig 31) Very soon after birth the cells in the body however began to lose their chromaffinity Already 3 days after birth when morphological changes could not be seen in the cells the body no longer gave a uniform reaction (Fig 32) in places even completely non chromaffin areas could be seen During the first postnatal week the intensity of the chromaffin reaction gradually decreased (Fig 33) and the amount of chromaffin positive tissue diminished In 7 day old rats only small faintly chromaffin cell groups were found in the body (Fig 34) and during the second week disappearance of the chromaffin tissue went further (Fig 35) In one month old rats chromaffin cells were not usually found in this region (Fig 36) However in one adult rat a small group of faintly chromaffin cells was found in the retroperitoneal region formerly occupied by the main para aortic body

The smaller para aortic bodies also lost their chromaffinity soon after birth and only very seldom chromaffin cells were found in them at the end of the first week after the birth

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Transverse sections through the middle part of the main para aortic body Dichromate formol fixation Frozen sections at 90 micra x 150

Ao = aorta Vc = vena cava S = sympathetic tissue Cr = chromaffin tissue bl = blood

Fig 29 — 16-day old fetus The body gives a faint just visible but homogenous chromaffin reaction

Fig 30 — 21 day old fetus Body forms a uniform structure Intensity of the chromaffin reaction has increased and the body has become enlarged

Fig 31 — Newborn rat The chromaffin reaction has still increased somewhat in its intensity and is uniform throughout the body

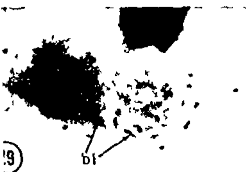
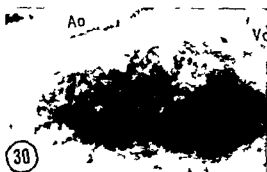
Fig 32 — 3 day old rat In places especially in the periphery of the body the intensity of the chromaffin reaction has clearly weakened

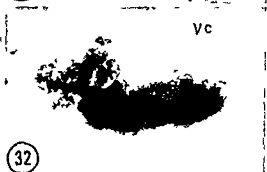
Fig 33 — 5 day old rat Gradual loss of chromaffinity can be noticed in the body In the lateral aspects of the body completely non-chromaffin areas are seen

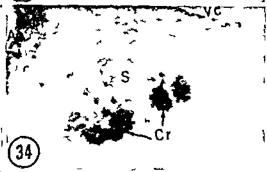
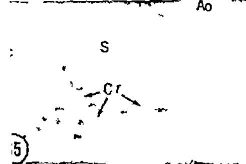
Fig 34 — 7 day old rat Most of the chromaffin tissue has disappeared from the body Intensity of the chromaffin reaction has continuously decreased Small faintly chromaffin cell groups are seen but large non-chromaffin areas dominate

Fig 35 — 9 day old rat The body is now almost completely non-chromaffin Isolated extremely weakly chromaffin cells are still seen in the body

Fig 36 — 30 day old rat Region formerly occupied by the main body Chromaffin cells are not seen.


bl
29

Ao
Vc
30

Vc
31

Vc
32

S
Vc
33

Vc
S
Cr
34

Ao
S
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35

S
bl
36

In fetuses aged 16 days, adrenal cortical cells formed an oval encapsulated mass which protruded somewhat into the dorsal coelomic cavity and on its medial lower aspects was closely related to the sympathetic elements primary sympathetic cells and fibres At this stage signs of migration of the primitive sympathetic cells from the nearby ganglia toward the cortical cells could be noticed (Fig 37) From this age onwards, small groups of cells with small, round and dark nuclei and scanty cytoplasm and also cells with characteristics of phaeochromoblasts were found in the middle of the cortex (Fig 38) These medullary cell islands gradually increased in size and number and the cells in them underwent further differentiation At the end of the fetal development most of the medullary cells were either phaeochromoblasts or mature chrom affin cells but still more primitive cells with dark and round nuclei were present (Fig 39)

In the newborn rat the adrenal medulla had not yet formed a compact structure but cortical cell cords were seen running among the medullary cell islands which were scattered around capillaries in the middle of the gland

Medullary cells of a full term fetus (21 days after copulation) exhibited a very faint chromaffin reaction In the newborn rat the reaction was still very weak approximately corresponding to that seen in the para aortic bodies of 17—18 days old fetuses (Fig 40) In the course of a few days the chromaffin cell groups fused together and the intensity of the chromaffin reaction increased (Fig 41) After a week the medullary tissue was distinctly separated from the cortical tissue and the cells had reached their full chromaffinity

ABDOMINAL AND LUMBAR SYMPATHETIC GANGLIA

While the para aortic bodies already gave a positive chromaffin reaction (16—17 days after copulation) the cells in the developing sympathetic ganglia were still at a more primitive stage of differentiation Cells with dark small and round nuclei and scanty cytoplasm predominated However more developed sympathetic cells with larger and paler nuclei (sympathicoblast) were already seen especially in the future coeliac ganglion (Fig 4[illegible]

Toward the birth sympathicoblasts underwent fu[illegible]r differentiation characterized by a rapid increase in the size of [illegible]lei and

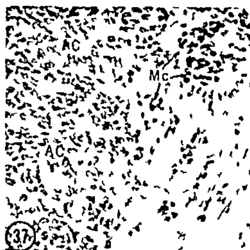


Fig 37 — 16 day old fetus Migrating primary sympathetic cells (Mc) from the adjacent sympathetic ganglia toward the adrenal cortex (AC) van Gieson x 250



Fig 38 — 17 day old fetus Small groups of future medullary cells (Mc) in the middle of the adrenal cortical cells (AC) van Gieson x 250

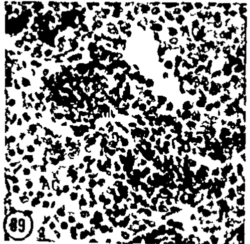


Fig 39 — 21 day old fetus Medullary cell groups have increased in size and have begun to fuse together forming the adrenal medulla (AM) At this stage the *majority of the medullary cells are still* phaeochromoblasts but numerous morphologically mature chromaffin cells can be observed van Gieson x 250

In fetuses aged 16 days adrenal cortical cells formed an oval encapsulated mass which protruded somewhat into the dorsal coelomic cavity and on its medial lower aspects was closely related to the sympathetic elements, primary sympathetic cells and fibres At this stage signs of migration of the primitive sympathetic cells from the nearby ganglia toward the cortical cells could be noticed (Fig 37) From this age onwards small groups of cells with small, round and dark nuclei and scanty cytoplasm and also cells with characteristics of phaeochromoblasts were found in the middle of the cortex (Fig 38) These medullary cell islands gradually increased in size and number, and the cells in them underwent further differentiation At the end of the fetal development most of the medullary cells were either phaeochromoblasts or mature chrom affin cells, but still more primitive cells with dark and round nuclei were present (Fig 39)

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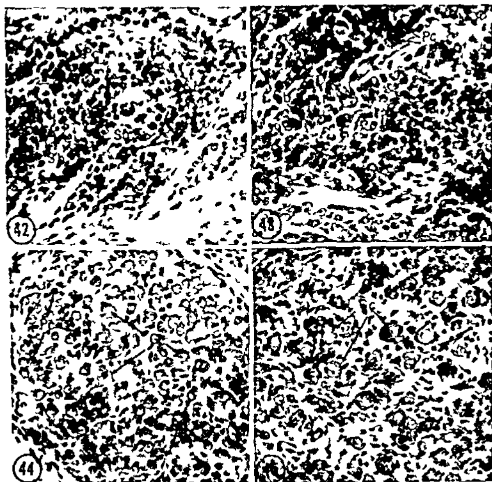


Fig 42 — 18 day old fetus Coeliac ganglion Majority of the cells in the ganglion are either young sympathicoblasts with large and pale nuclei (Sb) or more primitive cells with moderately basophilic small nuclei (Pc) Van Gieson x 250

Fig 43 — 21 day old fetus Coeliac ganglion Nuclei of the developing sympathicoblasts (Sb) have enlarged and there is also a clear increase in the amount of cytoplasm Mature ganglion cells are not observed Cells are closely packed together and nerve fibres are still poorly developed Numerous cells with small and dark nuclei (Pc) can be seen Van Gieson x 250

Fig 44 — 1 day old rat Coeliac ganglion Mature ganglion cells (Gc) are present with occasional mantle cells around them however developing sympathicoblasts (Sb) are numerously seen There is a clear increase in nerve fibres Cells with small dark and round nuclei are still present (Pc) but their number has decreased Van Gieson x 250

Fig 45 — 8 day old rat Coeliac ganglion Cells in the ganglion are dominantly mature ganglion cells with mantle cells around them Van Gieson x 250

Sympathetic ganglia were not all at the same stage of differentiation. Small dark polynucleolar cells and young sympathicoblasts were still numerously seen in the suprarenal ganglia at a time when the other ganglia were already matured (Fig 46). Also the superior mesenteric ganglion and the lumbar paravertebral ganglia were at birth slightly more primitive than the coeliac ganglion but they matured soon after birth.

Cells with nuclear characteristics of chromaffin cells were regularly found in the sympathetic ganglia of the newborn rat. These cells were especially to be seen in the suprarenal ganglia, in the superior mesenteric ganglion and in the lumbar ganglia. In the other sympathetic ganglia only occasional chromaffin cells were found. When the ganglia increased in size it was very difficult to follow the fate of the isolated chromaffin cells in them. Both the abdominal and lumbar sympathetic ganglia were completely non-chomaffin already 4—5 days after birth.

Cytoplasm of the adrenal medullary and the extra-adrenal chromaffin cells stained dark granular with Bodian's Protargol impregnation technique (Fig 47). These argyrophilic cells were also found in the prevertebral and paravertebral ganglia (Fig 48). However their number was small and they gradually disappeared with age and were seldom found in the ganglia after the first postnatal week.



Fig 46 — 6-day-old rat. Suprarenal ganglion. Numerous developing sympathicoblasts (Sb) are still observed in this ganglion. Cells with small basophilic nuclei (Pc) are also present. Cells with nuclear characteristics of chromaffin cells (Cc) can also be found in the ganglion. van Gieson. x 200

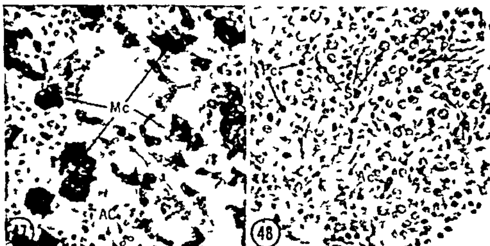


Fig 47 — Newborn rat Adrenal medulla Highly argyrophilic medullary cell islets (Mc) are seen scattered among the cortical tissue (AC) Bodian's Protargol impregnation x 250

Fig 48 — Newborn rat Superior mesenteric ganglion Numerous cells with dark small and round nuclei (Pc) are seen in the ganglion Nerve fibres are seen but they are still poorly developed Occasional argyrophilic cells (Ac) are observed Sb = sympathicoblasts Bodian's Protargol impregnation x 250

CERVICAL AND THORACIC GANGLIA

In every rat studied the paravertebral ganglia both in the cervical and the thoracic regions were completely non chromaffin

QUANTITATIVE STUDIES

VOLUME OF THE EXTRA ADRENAL CHROMAFFIN TISSUE

Because the vast majority of extra adrenal chromaffin tissue was confined to the compact para aortic bodies in which the cells were predominantly chromaffin it was possible to estimate planimetrically the approximate volume of that tissue from serial frozen sections

For practical reasons it was necessary to cut the sections as thick as 90 micra The faintly chromaffin cell islets were thus better distinguished when projected on to the paper for drawing Furthermore cutting thick sections made it possible to get complete series with relatively little variation in section thickness

It was impossible to measure the volumes of the extra adrenal chromaffin tissue with this method in animals 9 days of age or older By then only isolated faintly chromaffin cells were found

in the main para aortic body Furthermore the intensity of the chromaffin reaction was too weak for accurate drawing

The volume of the adrenal medulla was similarly recorded from the same series of sections As mentioned previously in the present paper, the adrenal medulla was composed of separate chromaffin cell islets in the first two postnatal days However the whole medullary area in each sections was measured and only the largest blood vessels were subtracted from the total volume Thus the values given were too large up to the third postnatal day when

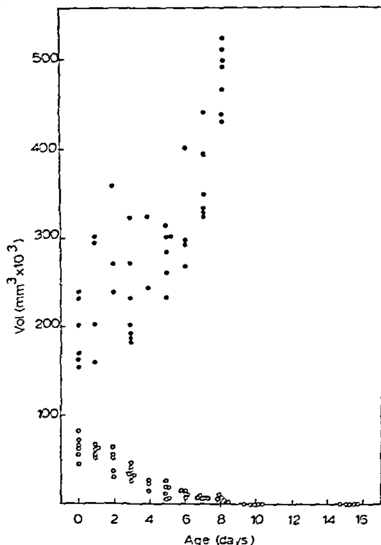


Fig 42 — Effect of age on the volumes of the adrenal medulla and the extra adrenal chromaffin tissue of the rat Circles indicate the individual values of the extra adrenal chromaffin tissue and dots those of the adrenal medulla

the medulla formed a compact distinctly separated homogenously chromaffin structure

The main results have been presented in Fig 49 It can be seen that already at birth a great majority of the chromaffin tissue was confined to the adrenal medulla However as previously mentioned in this chapter medullary volumes given are too large up to the third day so that in reality volumes of the intra and extra adrenal chromaffin tissues are more equal than illustrated in the figure

The medullary volume increased quickly during the first week after birth while that of the extra adrenal chromaffin tissue gradually decreased At 10 days of age the volume has been marked as a zero

Fig 50 shows the volume of the main para aortic body and of the chromaffin tissue outside it It is evident that the majority of the extra adrenal chromaffin tissue was confined to the main para aortic body Five days after birth planimetrically measurable chromaffin tissue outside the adrenals was present only in the main body

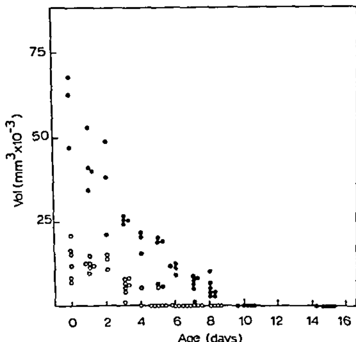


Fig 50 —Effect of age on the volumes of the extra adrenal chromaffin bodies of the rat Dots indicate the individual values of the main para aortic body and circles those of the smaller chromaffin bodies

DISCUSSION

As far as I am aware no previous studies have been made on the fetal extra adrenal chromaffin tissue of the rat

The observations of the present study indicate that the rat fetus does not in principle differ from the other animals studied Well defined chromaffin bodies develop in the vicinity of the abdominal pre aortic sympathetic plexuses and the formation and cellular differentiation of these bodies take place in the same way as described previously in other mammals (see the Review of the Literature)

The bulk of the extra adrenal chromaffin tissue was found in the main para aortic body The topography of this organ was almost exactly the same as that of the main para aortic body of the mouse as described by Coupland (1960) also the histological picture closely resembles that described in the mouse

The para aortic bodies of the rat were first seen to give a faint positive chromaffin reaction on the 16th day after copulation and the intensity of the chromaffin reaction gradually increased in them reaching its maximum at about the time of birth In the newborn rat the extra adrenal chromaffin tissue was already fully matured cells in the bodies were dominantly healthy looking chromaffin cells supported by a thin reticular type of connective tissue stroma These observations are in keeping with the findings made on other mammals (see the Review of the Literature)

The postnatal fate and degeneration of the extra adrenal chromaffin tissue in man and in some other mammals have been a subject of a brief discussion previously in this paper (see pages 14 and 15) It is generally accepted that there are certain species dependent differences in the postnatal fate of that tissue While the main bodies of some mammals *e g* the guinea pig and the rabbit are rather permanent organs in others *e g* man and the mouse they undergo degenerative changes during the early pre pubertal phase and most of the extra adrenal chromaffin cells finally disappear

Coupland (1954 b 1956 1960) has suggested that the differential growth of the sympathetic elements and the chromaffin bodies attached to them results in mechanical forces sufficient to disrupt the chromaffin bodies in man and in the mouse in which the connective tissue stroma is minimal in the body whereas in the

rabbit and the guinea pig the supporting connective tissue is sufficiently abundant to prevent this dispersal

In the present work the post natal fate of the main para aortic body of the rat was found to be in principle very much like that of the main body of the mouse as described by Coupland (1960) In both species the main body disintegrates fairly soon after the birth and most of the extra adrenal chromaffin cells disappear In the mouse Coupland found a more wide spread degeneration of the extra adrenal chromaffin cells after the disintegration of the main para aortic body and it was suggested by him that these changes are the result of the mechanical dispersal of the chromaffin cells However in the rat a marked decrease in the chromaffinity and wide spread signs of cellular degeneration were in the present study found in the chromaffin cells of the main para aortic body soon after the birth when the body had still kept its compact structure Nuclear irregularity and pyknosis and cytoplasmic vacuolization were prominent features in the body at the end of the first postnatal week The changes gradually increased and during the second week only a few healthy looking chromaffin cells were found in the body

Disappearance of the chromaffinity as well as cellular degeneration were also observed in the smaller para aortic bodies in which mechanical forces if any would be expected to be small

Though it is more difficult to follow the final fate of the isolated chromaffin cells in the growing sympathetic ganglia it seems clear after the present observations that also at least the vast majority of these cells disappeared fairly soon after the birth

In principle the present observations are in keeping with the results made previously in man *e g* by Scheel (1908) Handschin (1928) and Iwanow (1930 1932) It appears clear that the changes seen in the main para aortic body of the rat are degenerative rather than due to variations in the secretory activity of the chromaffin cells as proposed by Coupland (1960)

As was previously described in the present paper (see page 18) most of the earlier investigators who studied the extra adrenal chromaffin tissue of the rat used adult animals and failed to find chromaffin tissue outside the adrenals In view of the present observations this is not surprising However Coupland (1960) examined two 1 day old rats after dichromate fixation but did not find any extra adrenal chromaffin tissue This is somewhat surpri

sing, since in the present study the main para aortic body was always easily found in 1—7 days old rats Strain dependent differences in this tissue in the rat is a possible explanation for the discrepancies though not a very likely one Coupland used Wistar rats while descendants of the Sprague Dawley strain were used in the present study

The extension of primitive sympathetic elements into the adrenals is well recognized Pankratz (1931) observed that in the rat fetus migration of the primary sympathetic cells toward the cortex begins on about the 16th day and continues probably till birth These observations are in agreement with the present work In the present study, medullary cells were first seen to give a faintly positive chromaffin reaction at the end of the fetal life (20—21 days after copulation) This is in keeping with the observations made by Smitten (1963) During the first postnatal day the intensity of the chromaffin reaction in the medulla was still weaker than that of the extra adrenal chromaffin tissue These observations indicate that in the rat the extra adrenal chromaffin tissue functions as a main source of catecholamines during the fetal life However during the first postnatal week the medulla reaches its maturity while the bulk of the extra adrenal chromaffin tissue disappears After that practically all the chromaffin tissue of the rat is confined to the adrenal glands In principle this is in keeping with the observations made *e g* in man and the mouse but in the rat the postnatal disappearance of the extra adrenal chromaffin tissue takes place more rapidly and is more wide spread

PART II

EXPERIMENTAL STUDIES

EARLIER EXPERIMENTAL INVESTIGATIONS

The pathology particularly neoplasms of the chromaffin tissue has been the subject of a large number of investigations It is not possible in the present study to go deeply into this voluminous literature (for ref see for ex Wiesel 1904, Gierke 1906 Herxheimer 1914 Handschin 1928 Mackeith 1944 Palmieri Ikkos & Luft 1961) On the other hand experimental studies dealing with the chromaffin tissue have almost without any exception been concerned with the adrenal medulla and as far as I know, there are only 4 publications in which the extra adrenal chromaffin tissue has been experimentally studied

Borberg (1913) studied the extra adrenal chromaffin tissue of the rabbit under various experimental conditions He examined the effect of various chemicals (*e.g* pilocarpin choline) metals (P, As Pb) bacterial toxins (botulismus tetanus) infections tissue extracts (thyreoidea pancreas placenta) anaemia asphyxia hemiadrenalectomy *etc* on the chromaffinity of the extra adrenal chromaffin tissue The results however were very variable and it was not possible to draw any distinct conclusion about them No specific changes in the chromaffinity of the para aortic bodies of the experimental animals were noticed

Wislocki & Growe (1922 1924) destroyed the adrenals of the dog both surgically and by using radium implants and then studied the chromaffinity of the main para aortic body They did not find any changes due to adrenalectomy in the chromaffin reaction of the main para aortic body Destruction of both the adrenal medulla and the extra adrenal chromaffin tissue did not produce any clinical symptoms in the dog

Goormaghtigh (1936) performed double adrenalectomy in the mouse The intensity of the chromaffin reaction in the chromaffin sympathetic paraganglionic tissue in these adrenalectomized animals did not differ from the controls and no compensatory hypertrophy of the extra adrenal chromaffin tissue could be demonstrated

PRELIMINARY STUDIES

The primary purpose of these studies was to find out whether bilateral adrenalectomy performed sufficiently early during the first postnatal days, had any effect on the normally rapid disappearance of the extra adrenal chromaffin tissue of the rat

For this purpose newborn rats were adrenalectomized and substitution therapy was given by daily injections of corticosteroids (cortisone and DOCA) and NaCl The animals were then killed at an age when normally only rudiments of the extra adrenal chromaffin tissue was left (7—11 days after birth)

The results were striking Regenerates of adrenal tissue (both cortical and medullary) were common However in all the operated animals studied (15 rats) whether medullary and cortical tissue was found or not the amount of the extra adrenal chromaffin tissue clearly exceeded that of the normal controls of the same age In one group (5 rats of 7—8 days) the planimetrically measured volume of the extra adrenal chromaffin tissue was larger than that of normal newborn

In the following control studies the animals were adrenalectomized during the third day after birth to avoid regeneration of the adrenals At this age the adrenal capsule was strong enough to permit removal of the glands without tearing them To control the possible effect of the corticosteroids used in the substitution therapy the animals were now divided into two groups One group was injected daily with cortisone and DOCA as previously the other group only with DOCA and NaCl The animals were then killed between the age of 11—13 days It turned out that the extra adrenal chromaffin tissue had in a normal way completely disappeared in the group which had received only DOCA as a substitution (8 rats) while in the cortisone group (only 3 rats survived) most of the extra adrenal chromaffin tissue was left

It was then evident that removal of the adrenal medulla does not exert any influence on the extra adrenal chromaffin tissue On the other hand it was thought very likely that cortical steroids especially cortisone prevents the disappearance of that tissue

EFFECT OF ADRENOCORTICAL HORMONES AND ACTH ON THE CHROMAFFIN TISSUE

EARLIER INVESTIGATIONS

It has been accepted that medullary catecholamines cause an increased secretion of cortical hormones (for ref see f ex Vogt 1954 Ross 1961) However no direct evidence is available of the influence of cortical hormones on chromaffin cells

The number of investigations dealing with the effect of cortisone or other cortical steroids on the adrenal medulla is small So far as I am aware there are no previous reports about the effects of exogenously given cortical hormones on medullary chromaffin cells Eränkö (1952) did not find any changes in the adrenal medullary cells after 5 days stimulation of large doses of ACTH into rats Feldman (1951) showed a statistically significant decrease in the medullary volume of rats treated with ACTH for 5 days Hokfelt (1951) and Eränkö (1952) observed a significant decrease in the adrenalin content of the medulla in the rat after 5—10 days ACTH treatment while the noradrenalin content showed no clear differences from normal

I have found no published work at all on the effect of cortical steroids on the extra adrenal chromaffin tissue

MATERIAL AND METHODS

Normal rats from newborn to 15 days after birth were used as experimental animals Most of the animals in each experimental group were litter mates All were freely taken care of by mothers

The material comprised about 115 cortisone treated rats 35 hydrocortisone treated 16 ACTH treated and 7 DOCA treated rats Further details will be presented later in appropriate connexion

Experimental animals injected with cortisone or hydrocortisone did not survive when kept together with normal untreated controls because the mothers used to abandon the weaker experimental animals It was therefore necessary to take controls from different litters The normal control material presented

in Part I was collected from amongst the same groups of pregnant rats from which the litters used for injection experiments were taken

Most of the experimental animals were used for quantitative studies in which the volumes of the adrenal medulla and extra adrenal chromaffin tissue were measured planimetrically from a complete series of frozen sections cut at 90 micra after dichromate formol fixation as previously described As also in the normal material (Part I) both encapsulated chromaffin bodies and non encapsulated groups of chromaffin cells giving a homogenous chromaffin reaction in the abdominal and lumbar sympathetic ganglia were measured

Some of the sections were in addition examined using Schmorl s ferro-ferricyanide technique

For histological studies Bodian fixed paraffin sections were stained using both the van Gieson method and Bodian s Protargol impregnation technique

A tissue block containing both cervical and thoracic sympathetic ganglia was taken from 10 hydrocortisone treated animals These blocks were then systematically studied after dichromate fixation Some of these sections were stained with Schmorl s ferro ferricyanide technique

RESULTS

GENERAL STATE OF THE EXPERIMENTAL ANIMALS

Experimental animals in cortisone and hydrocortisone groups gained in weight very slowly during hormone administration The skin of the animals grew loose wrinkled and scurfy and it became brownish The hairs were long but thin and shaggy and a little tinged with gray Haemorrhagic enteritis and diarrhoea were common However most of the animals were vigorous and lively and the general development (opening of the eyes *etc*) took place normally

These effects were more evident in the animals treated with large doses of cortisone and hydrocortisone for a longer time from the first days after birth With smaller doses and after a shorter duration of the treatment fewer symptoms were observed

Animals treated with large doses of ACTH closely resembled those treated with smaller doses of cortisone

DOCA even in large doses seemed to be without any effect on the weight or general state of the animals

Weights of the animals have been presented diagrammatically in connexion with the adrenal medullary volumes elsewhere in the present paper (Figs 54—56 and 58)

QUANTITATIVE STUDIES

1 EFFECT OF CORTISONE AND HYDROCORTISONE ON THE CHROMAFFIN TISSUE

EXPERIMENTAL

A total number of 91 cortisone and 30 hydrocortisone treated rats were studied.

Aqueous suspension of cortisone and hydrocortisone acetate (ADRESON and HYDROADRESON N V ORGANON OSS HOLLAND) were given subcutaneously as a daily injection in 0 1 ml of physiological NaCl

1st cortisone group (60 rats) The injections were initiated during the first 12—36 postnatal hours The following experiments were made

— 0.25 mg cortisone daily for 4—12 days (29 rats)
— 0 125 mg for 9—10 days (9 rats)
— 0 125 mg daily for 4 days then 0 25 mg for 10 days (6 rats)
— 0.25 mg every other day for 6—7 days (10 rats)
— 0 125 mg every other day for 9—10 days (6 rats)

2nd cortisone group (9 rats) 0.25 mg cortisone daily from the third day after birth for 9 days

3rd cortisone group (6 rats) 0 5 mg of cortisone daily from the 5th day after birth for 5 days then 0 75 mg for 3—4 days

4th cortisone group (5 rats) 0 75 mg cortisone daily from the 7th day after birth for 8 days

5th cortisone group (5 rats) 2 mg cortisone daily from the 10th day after birth for 5 days then for 3 days 1 75 mg daily

6th cortisone group (6 rats) 2 5 mg cortisone daily from the 15th day after birth for 3 days then 2 0 mg daily for 5 days

If the administration of cortisone was initiated after the third postnatal day the dose was calculated so that the daily dosage of cortisone per gm of body weight (means of weights in each age group) exceeded the largest dose used for the younger ones (0 25 mg daily or about 0 04 mg cortisone per gm of body weight daily)

Hydrocortisone group (30 rats) Hormone administration was initiated during the first 24—36 postnatal hours The following dosages were used

— 0.5 mg daily for 8—12 days (5 rats)
— 0 25 mg daily for 7—13 days (10 rats)
— 0 125 mg daily for 7—9 days (15 rats)

RESULTS

Volume of the Extra adrenal Chromaffin Tissue

Fig 51 shows that the total volume of extra adrenal chromaffin tissue of the rats repeatedly injected with 0 25 mg of cortisone from the first two days after birth significantly increased during hormone administration The effect was already clear after 2—3 days treatment but the volume gradually increased when hormone injections were continued for longer periods

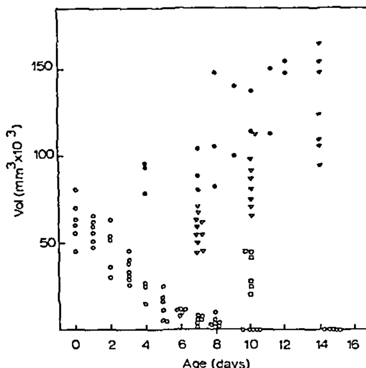


Fig 51 — Effect of repeated injections of cortisone on the volume of the extra adrenal chromaffin tissue of the rat Cortisone administration initiated during the first 12—36 postnatal hours Each point represents one animal ● = 0.25 mg cortisone daily ▼ = 0 125—0 25 mg daily ▽ = 0 125 mg daily or 0 25 mg every other day □ = 0 125 mg every other day O = untreated controls Difference between normal controls and cortisone treated animals highly significant ($P < 0.001$)

0 125 mg cortisone daily (or 0 25 mg every other day) completely prevented the disappearance of extra adrenal chromaffin cells and was sufficient to cause a significant increase in the volume when given for longer periods

Disappearance of the extra adrenal chromaffin tissue took place in animals receiving only 0 125 mg of cortisone every other day however also in this group at the age when normally no or only occasional extra adrenal chromaffin cells were present comparatively large amounts of chromaffin tissue were found outside the adrenals

It can be seen from Fig 52 that hydrocortisone had an influence on the extra adrenal chromaffin tissue similar to that of cortisone However, the increase in the volume due to hydrocortisone administration was stronger than that caused by equal doses

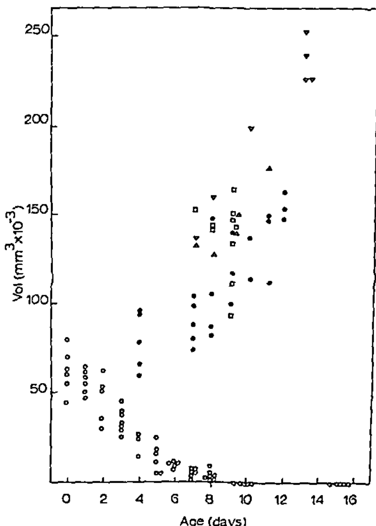


Fig 52 — Effect of repeated injections of hydrocortisone on the volume of the extra adrenal chromaffin tissue of the rat Hydrocortisone administration initiated during the first 24—36 postnatal hours △= 0 5 mg hydrocort daily ▽= 0 25 mg hydrocort daily □ = 0 1?5 mg hydrocort daily ● = 0 2? mg cortisone daily O = untreated controls Difference between normal controls and hydrocortisone treated animals highly significant ($P < 0\ 001$)

of cortisone The effect was maximal with a daily dose of 0 25 mg of hydrocortisone and further increase of the dosage did not further increase the volume of the extra adrenal chromaffin tissue

Fig 53 shows that the effect of cortisone on the extra adrenal chromaffin tissue closely depended on the age of animals at the beginning of hormone administration The later the cortisone injections were initiated the smaller was the volume increase

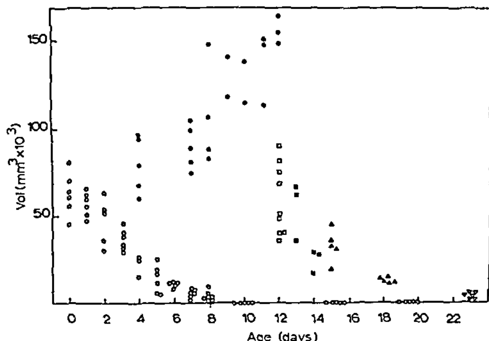


Fig 53 — Effect of cortisone and the age of animals at the beginning of the hormone treatment on the volume of the extra adrenal chromaffin tissue O = untreated controls ● = 0 25 mg cortisone from the first day □ = cortisone from the 3rd day ■ = cort from the 5th day △ = cort from the 7th day ▲ = cort from the 10th day ▽ = cort from the 15th day after birth The effect of cortisone is smaller if the administration is begun later

Volume of the Adrenal Medulla

Fig 54 shows that the volume of the adrenal medulla of normal untreated rats gradually increased when the animals were gaining in weight

Animals injected with cortisone gained in weight very slowly or not at all and the volumes of the adrenal medulla of these rats although smaller in absolute terms were of the same relative value in terms per gm of body weight as those of normal controls

Hydrocortisone exerted an influence similar to that of cortisone (Fig 55)

Fig 56 illustrates the effect of cortisone on the volume of the adrenal medulla when hormone administration was initiated later during the postnatal life A slight tendency towards smaller relative volumes is noticeable

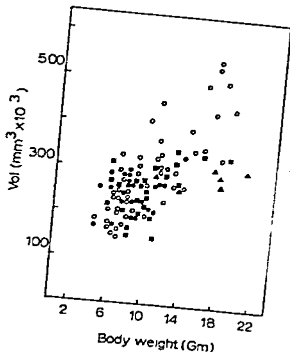


Fig 54

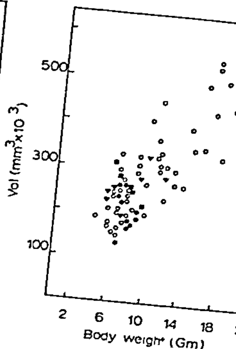


Fig 55

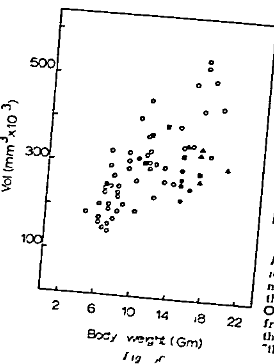


Fig 56

Fig 54 — Effect of cortisone on the body weight and on the volume of the adrenal medulla Repeated injections of cortisone initiated during the first 12—36 hours Each point represents one animal O = untreated controls ● = 0.25 mg cortisone daily ■ = 0.125—0.25 mg daily ▲ = 0.125 mg every other day

Fig 55 — Effect of hydrocortisone on the body weight and on the volume of the adrenal medulla Hormone injections initiated during the first 24—36 postnatal hours O = untreated controls ■ = 0.5 mg hydrocortisone daily ▲ = 0.25 mg daily ● = 0.125 mg daily

Fig 56 — Effect of cortisone administration initiated later during the first postnatal week on the body weight and on the volume of the adrenal medulla O = untreated controls ● = cortisone from the 3rd day ■ = cortisone from the 5th day ▲ = cortisone from the 7th day

2 EFFECT OF DOCA ON THE CHROMAFFIN TISSUE

EXPERIMENTAL

Retroperitoneal tissue blocks of 7 DOCA treated animals were studied

0 005 mg (3 rats) or 0 05 mg (4 rats) desoxycorticosterone acetate (PRIMO CORT LEIRAS FINLAND) was given daily from the first day after birth for a week

RESULTS

Volume of the Extra adrenal Chromaffin Tissue

Fig 57 shows that the disappearance of extra adrenal chromaffin tissue of DOCA treated animals took place normally without regard to the dose At the end of treatment (7 and 8 days old) only small faintly chromaffin cell groups were left in the main para aortic body

Volume of the Adrenal Medulla

Animals treated with DOCA gained in weight normally and the volumes of the adrenal medulla corresponded with those of normal ones of the same weight (Fig 58)

3 EFFECT OF ACTH ON THE CHROMAFFIN TISSUE

Because the changes observed in the extra adrenal chromaffin tissue after cortisone and hydrocortisone treatment concern the pharmacological action rather than the physiological action of cortisone and hydrocortisone it was interesting to see whether the adrenal cortex of young rats could be stimulated to produce enough corticosteroids to exert an influence similar to that of exogenously given cortisone

EXPERIMENTAL

Sixteen rats were studied Administration of hormone was initiated during the second day after birth ACTH was given daily as a subcutaneous injection (ACORTAN PROLONGATUM FERRING SWEDEN 40 I U per ml 1961)

In the first group the daily dose was 0 5 I U for 5 days after which 1 0 I U was given daily for 3 days

In the second group 2 0 I U (0 05 ml undiluted preparate) was given daily for 7 days and in the third group the dose of ACTH was 8 I U (0 2 ml undiluted preparate) daily for 7 days

Volume of the Extra adrenal Chromaffin Tissue

Fig 57 shows that massive doses of ACTH (8 I U daily) completely prevented the disappearance of the extra adrenal chromaffin tissue but did not cause any increase in the volume of that tissue

Daily dose of 2 I U exerted a slight influence Although the volume of the extra adrenal chromaffin tissue decreased in this group the volume in 8 day old ACTH treated rats clearly exceeded that of the normal controls of the same age

In the group receiving only 0 5—1 0 I U of ACTH daily the disappearance of the extra adrenal chromaffin cells took place normally

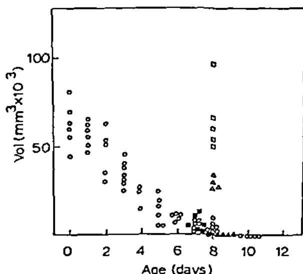


Fig 57 — Effect of repeated injections of DOCA and ACTH on the volume of the extra adrenal chromaffin tissue O = untreated controls ■ = DOCA treated animals (0 005 mg or 0 05 mg daily) □ = 8 I U of ACTH daily △ = 2 I U of ACTH daily ▲ = 0 5—1 0 I U of ACTH daily There are no significant differences between normal controls and animals injected with DOCA or the lowest dosage of ACTH Difference between controls and rats daily treated with 2 or 8 I U of ACTH is highly significant (P > 0 001)

(Fig 58)

Animals injected daily with 2 I U of ACTH gained in weight normally and the volumes of the adrenal medulla did not differ from those of normal controls of the same weight

The gain in weight was somewhat slower in the group treated with 8 I U of ACTH In this group the medullary volumes were clearly larger than those in the previous ACTH group and even larger than those of the normal untreated rats of the same weight

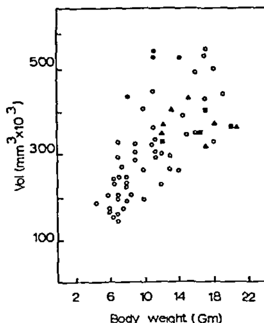
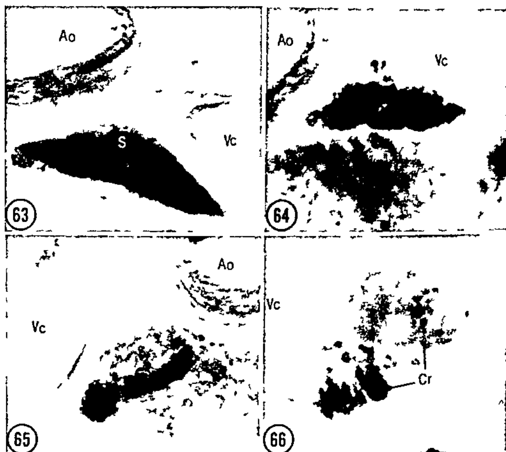


Fig 58 — Effect of DOCA and ACTH on the body weight and on the volume of the adrenal medulla O = untreated controls ● = 8 I U of ACTH daily ■ = 2 I U of ACTH daily ▲ = DOCA treated animals



Transverse sections throught the middle part of the main para aortic body Dichromate formol fixation Frozen sections at 90 micra x 130 Ao = aorta Vc = vena cava S = sympathetic tissue Cr = chromaffin tissue

Fig 63 — 12 day old cortisone treated rat Cortisone injections initiated during the 3th day after birth The body has elongated and flattened but has kept its compact structure Chromaffin reaction is strong and uniform throughout the body Chromaffin tissue is seen among the sympathetic tissue in the dorsal aspect of the body

Fig 64 — 14 day old cortisone treated rat Cortisone injections initiated from the 5th day after birth The body has gradually flattened Strong uniform chrom affin reaction is observed throughout the body

Fig 65 — 15 day old cortisone treated rat Cortisone injections initiated from the 7th day Owing to the gradual elongation the body has further flattened Sympathe tic tissue has increased Strongly chromaffin cell groups can be seen in the body and chromaffin c lls are observed also in the ganglia in the dorsal side of the body

Fig 66 — 18-day old cortisone treated rat Cortisone injections initiated from the 10th day Th body has lost its compact structure Clearly chromaffin cell groups can be seen in the region formerly occupied by the body and also in the nearby sympathetic ganglia



Fig 67 — Main para aortic body of an 8 day old ACTH treated rat (8 I U daily from the second day after birth) The body gives a uniform chromaffin reaction The intensity of the chromaffin reaction approximately corresponds to that seen in the body of normal newborn Formol dichromate fixation Frozen section at 90 micra x 150

Fig 68 — Main para aortic body of an 8 day-old DOCA treated rat (0 05 mg DOCA daily from the first day after birth) the bulk of the chromaffin tissue has disappeared from the body Only a few faintly chromaffin cell groups (Cr) are observed Formol dichromate fixation Frozen section at 90 micra x 150

Smaller Chromaffin Bodies and Sympathetic Ganglia

In animals treated with hydrocortisone and with larger doses of cortisone from the first two days after birth not only was the disappearance of extra adrenal chromaffin tissue prevented but chromaffin tissue appeared in abundance in structures such as the sympathetic ganglia in which normally no or only occasional chrom affin cells were seen This new chromaffin tissue was found in all sympathetic ganglia both in the prevertebral and paravertebral positions more numerously in the suprarenal ganglia in the superior mesenteric ganglion and in the lumbar paravertebral ganglia Especially numerous chromaffin cells were seen in the periphery of the ganglia near the extra adrenal chromaffin bodies which had remained undegenerated and were usually continuous with the chromaffin tissue inside the ganglia As a rule the chromaffin cell cords continued from the suprarenal ganglia into the adrenal medulla connecting the intra and extra adrenal chromaffin tissues (Figs 69—74 77 78 and 84)

LG

LG

Cr

(70)

(71)

(72)

Cr

Fig 69 — Lumbar ganglia of a 10 day old cortisone treated rat (0.25 mg cortisone from the first day after birth) A transverse section through the level of the main para aortic body The ganglia (LG) are full of clearly chromaffin tissue Formol dichromate Frozen section at 90 micra x 150

Fig 70 — Lumbar ganglia of a normal newborn rat (Same ganglia as in fig 69) The bulk of the tissue in these ganglia (LG) is non-chromaffin A small faintly chromaffin cell group (Cr) is observed Formol dichromate Frozen section at 90 micra x 150

Fig 71 — 14-day old cortisone treated rat (0 25 mg cort from the first day) A transverse section through the level of the origin of the superior mesenteric artery A strongly chromaffin body (CB) is seen in the vicinity of the coeliac ganglion The body has enlarged and the intensity of the chromaffin reaction increased Chromaffin tissue is seen abundantly also inside the ganglion (CG) Formol dichromate Frozen section at 90 micra x 150

Fig 72 — 10 day old hydrocortisone treated rat Suprarenal ganglion (0 25 mg The coeliac ganglion (CG) is completely non chromaffin A small para aortic body (CB) gives a uniform chromaffin reaction but its intensity is weaker than that in the body seen in fig 71 Formol dichromate Frozen section at 90 micra x 150

Fig 73 — 10 — day old hydrocortisone treated rat Suprarenal ganglia (0.25 mg hydrocortisone daily from the first day) Chromaffin tissue is seen in abundance in the ganglia (SGR) more plentifully in the periphery Chromaffin cell cords (Cr) are observed to continue from the ganglia towards the adrenal glands connecting the intra and extra adrenal chromaffin tissues Formol dichromate Frozen section at 90 micra x 150

Fig 74 — Suprarenal ganglion of a normal newborn rat Small groups of faintly chromaffin cells are seen in the periphery of the ganglion which in itself is completely non chomaffin Adrenal medullary tissue (AM) continues to the surface of the gland but is not continuous with extra adrenal chromaffin tissue Formol dichromate Frozen section at 90 micra x 150

Morphologically the ganglia developed and matured normally during cortisone and hydrocortisone treatment and the only clear difference was that the number of chromaffin cells markedly increased in them while it gradually decreased in untreated animals with age (Figs 75—78 and 79—82)

The smaller the dose of cortisone the fewer the chromaffin cells seen in the ganglia In the group given 0 125 mg of cortisone every other day extra adrenal chromaffin cells were *only occasion* ally seen outside the main para aortic body after a week s treatment

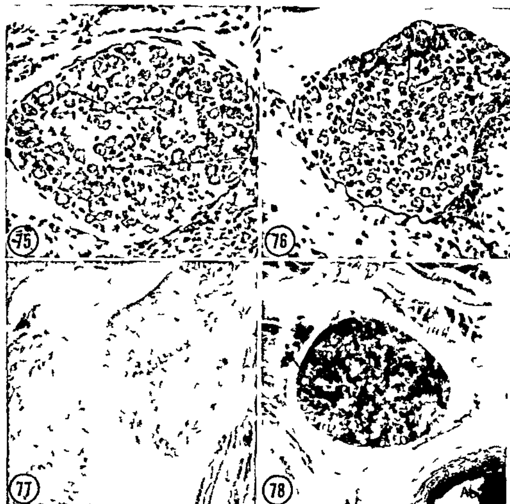


Fig 75 — Lumbar ganglion of a 10 day old normal rat The ganglion is morphologically mature Cells are mature ganglion cells with mantle cells around them Chromaffin cells are not observed van Gieson x 250

Fig 76 — Lumbar ganglion of a 10 day old hydrocortisone treated rat (0 125 mg hydrocortisone from the first day) Majority of the cells in the ganglion are mature ganglionic cells with mantle cells around them (Gc) Numerous cells with nuclear characteristics of chromaffin cells are observed in the ganglion (Cc) van Gieson x 250

Fig 77 — Lumbar ganglia of a 10 day old normal rat Formol dichromate fixation Schmorl s staining Frozen section at 40 micra x 200 The ganglia are completely non chromaffin Schmorl positive cells (chromaffin cells) are not observed

Fig 78 — Lumbar ganglion of a 10 day old hydrocortisone treated rat (0 125 mg hydrocortisone from the first day after birth) Formol dichromate fixation Schmorl s staining Frozen section at 40 micra x 200 Numerous isolated Schmorl positive cells (Cc) can be seen in the ganglion and also alongside the nerve fibres outside the ganglion

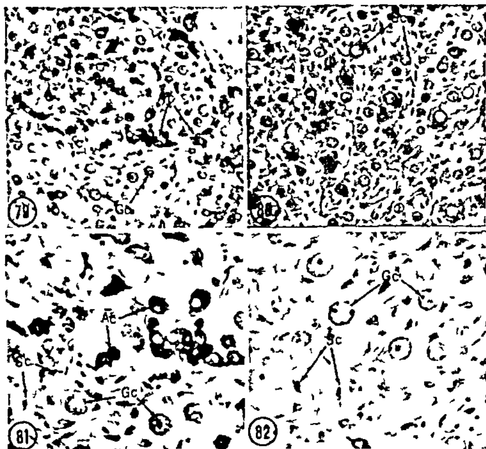


Fig 79 — Superior mesenteric ganglion of an 8 day old hydrocortisone treated rat (0.125 mg hydrocortisone daily from the first day after birth) Bodian's Protargol impregnation x 250 Numerous highly argyrophilic cells (Ac) can be seen in the ganglion Ganglion cells (Gc) are morphologically mature

Fig 80 — Superior mesenteric ganglion of an 8 day old untreated rat Bodian's Protargol impregnation x 250 Argyrophilic cells are not present

Fig 81 — Higher magnification detail (x 500) from fig 79 Cytoplasm of the argyrophilic cells is dark and granular nuclei are round and light and the cells show characteristics typical of the chromaffin cells Satellite cells (Sc) are regularly seen around ganglion cells (Gc)

Fig 82 — Higher magnification detail (x 500) from fig 80 Gc = ganglion cell Sc = satellite cell (mantle cell)

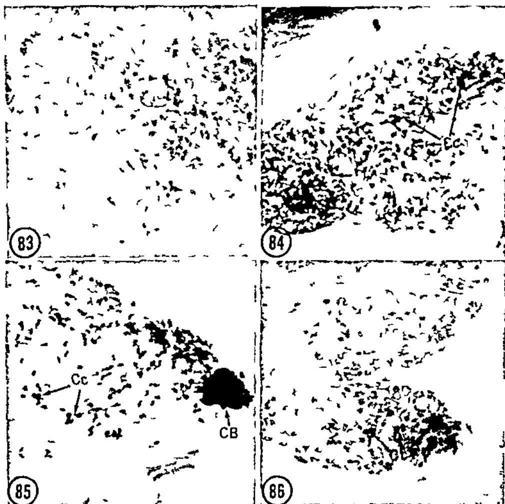


Fig 83 — Coeliac ganglion of a normal newborn rat Formol dichromate Schmorl s staining x 130 The ganglion is completely non chromaffin and Schmorl positive cells are not present

Fig 84 — Coeliac ganglion of a 10 day old hydrocortisone treated rat (0 125 mg hydrocortisone daily from the first day day after birth) Formol dichromate Schmorl s staining x 130 Numerous Schmorl positive cells (chromaffin cells) (Cc) are seen in the ganglion

Fig 85 — Coeliac ganglion of a 12 day old cortisone treated rat (Cortisone injections initiated from the 3th day after birth) Formol dichromate Schmorl s staining x 130 A small chromaffin body is seen in the vicinity of the ganglia in which numerous Schmorl positive cells (Cc) are observed more numerously near the chromaffin body

Fig 86 — Coeliac ganglion of an 8 day old ACTH treated rat (8 I U ACTH daily from the second day after birth) Formol dichromate Schmorl s staining x 130 A few Schmorl positive cells (Cc) are observed in the periphery of the ganglion Rest of the ganglion is completely chromaffin negative

The later cortisone administration was initiated in spite of the fact that large doses of cortisone were used the fewer the chromaffin cells appearing in the sympathetic ganglia In the group in which the hormone injections were started from the third day after birth the effect was still clear and numerous chromaffin cells were seen both in the abdominal and the lumbar sympathetic ganglia (Fig 85) In animals treated with cortisone from the 10th and 15th day after birth only occasional faintly chromaffin cells were found in the lumbar ganglia

Smaller doses of ACTH did not prevent the disappearance of chromaffin cells from the sympathetic ganglia However in the animals treated with 8 I U of ACTH for a week chromaffin cells were found both in the abdominal and lumbar ganglia approximately in the same degree as normally at birth (Fig 86)

DOCA was without any effect on the sympathetic ganglia and did not prevent the normal disappearance of the chromaffin cells in them

In animals treated with hydrocortisone chromaffin cells were also found in the cervical and thoracic paravertebral ganglia in which these cells were never seen in normal untreated animals (Fig 87)



Fig 87 — Superior cervical ganglion of a 12 day old hydrocortisone treated rat Formol dichromate Schmorl s staining x 130 Small groups of Schmorl positive cells (Cc) are observed in the ganglia

Cortisone and hydrocortisone had no clear effect on the adrenal medulla

Medullary cells gave a strong and uniform chromaffin reaction The intensity of the chromaffin reaction was approximately the same or slightly stronger than that of normal controls of the same age

In the animals treated with large doses of ACTH (8 I U daily) medullary cells gave a uniform reaction which however was somewhat weaker than that seen in the medulla of cortisone treated rats

LATER FATE OF THE EXTRA ADRENAL CHROMAFFIN TISSUE PRESERVED WITH CORTISONE

It was observed that repeated injections of cortisone and hydrocortisone prevented the postnatal disappearance of extra adrenal chromaffin tissue in the rat It was of obvious interest to follow up the later fate of that tissue after cessation of the hormone administration

EXPERIMENTAL

One month's experiment 0.25 mg of hydrocortisone was injected daily from the second day after birth for 9 days after which hormone administration was stopped From this group only 2 animals survived and they were killed at the age of 40 days

20-days and 30-days experiments 15 animals (from two litters) were treated daily with 0.125 mg of cortisone from the second day after birth for 7 days One animal was killed at the age of 8 days to check the effect No more cortisone was then given One group (8 rats) was sacrificed at the age of 31 days and the other a month later (60 days old)

RESULTS

Soon after cessation of hormone injections the animals reached normal controls in weight At the time of killing it was not possible to distinguish them from untreated animals of the same age

The main para aortic body of the hydrocortisone treated animals gave a strong and rather uniform chromaffin reaction Fibrotic tissue was increased in the body but it had kept its previous compact structure (Fig 88)

Smaller chromaffin bodies were seen in the vicinity of the abdominal sympathetic ganglia in which numerous isolated chrom affin cells were found

In the cortisone groups (31 and 60 days old) the main body had lost its compact structure but a considerable amount of strongly chromaffin tissue was found in the region formerly occupied by the main body (Fig 89) Small groups of chromaffin cells were also seen associated with the abdominal sympathetic ganglia and

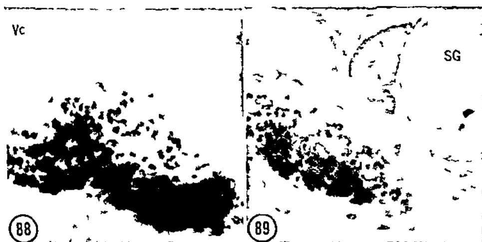


Fig 88 — Main para aortic body of a 40 day old hydrocortisone treated rat (0 ? mg hydrocortisone from the second day after birth for 9 days after which one month without any treatment) The body has kept its compact structure giving a strong chromaffin reaction Isolated chromaffin cells are observed in the dorsal side of the body among increased fibrous tissue Formol dichromate fixation 90 micra frozen section x 130

Fig 89 — Two month old cortisone treated rat (0 125 mg cortisone from the second day after birth for 7 days then without any treatment) This figure shows the region formerly occupied by the main para aortic body which has disintegrated Strongly chromaffin tissue (Cr) is observed in the region Formol dicromate fixation 90 micra frozen section x 130



Fig 90 — Superior mesenteric ganglion of a two month old cortisone treated rat (Hormone administration as in fig 89) Formol dichromate Schmorl s staining x 130 Numerous Schmorl positive cells (Cc) are observed in the ganglia

isolated chromaffin cells were seen both in the prevertebral and paravertebral sympathetic ganglia (Fig 90) Both the amount an the appearance of the extra adrenal chromaffin tissue were approximately the same in all cortisone treated animals without regard to the length of time after cessation of the treatment

DISCUSSION

DEGENERATION MECHANISM OF EXTRA ADRENAL CHROMAFFIN CELLS

Postnatal disappearance of the extra adrenal chromaffin tissue has been supposed to result through two possible mechanisms (see page 15)

1 Primary degeneration (Zuckerkandl 1912 Handschin 1928 Iwanow 1930 and others)

2 Mechanical forces produced by the differential growth of sympathetic nerve fibres and contiguous chromaffin elements (Coupland 1960) The results obtained in Part I of the present paper favour the view that in the rat the postnatal disappearance of the extra adrenal chromaffin cells is due to primary cell degeneration Since cortisone prevented the slight proliferation of the connective tissue in the normally degenerating chromaffin bodies presumably through its inhibiting effect on the growth of fibroblasts and formation of collagen (*e g* Chvapil 1959 Bush 1962 Kivirikko 1963) it thus would be able to reduce any passive disintegration of the chromaffin bodies due to fibrosis However

cortisone was not only able to prevent the disappearance of the extra adrenal chromaffin tissue, but also to cause an increase in the volume of chromaffin tissue This strongly suggests that the effect of cortisone is not based on inhibition of the connective tissue It rather appears that a sufficient concentration of cortisone may be necessary for the differentiation and survival of chromaffin cells

It is known that cortical cells of the rat adrenal begin to function about 19 1/2 days after fertilization (Josimovich *et al*, 1954) and that the hypophysis adrenal system also functions before birth (Kitchell & Wells 1952 a 1952 b Yakaitis & Wells 1956) However, as was observed in the present work (Part I), the chromaffin reaction appeared in the para aortic bodies before that already about 16 days after copulation This would seem to suggest that the chromaffin cells would be able to differentiate during fetal life in the absence of cortisone However it is possible that maternal steroids of which corticosterone and hydrocortisone are known to penetrate the placental barrier (Migeon *et al*, 1961) provide a sufficient corticoid level for the differentiation and survival of the fetal chromaffin cells

It has been observed that in the rat the volume of the adrenal cortex and especially the fasciculata markedly decrease immediately after birth (Josimovich *et al*, 1954) Moreover, it has been suggested that both the pituitary and the adrenal undergo alterations in activity during the early stages of postnatal d nt (Jailer 1950 Moog 1953) Thus some support is availa speculation that the blood level of circulating steroids is t birth support the extra adrenal chromaffin cells w te be corticoid production is sufficie of extra adrenal chromaffin ion remains unchanged after seem to support this view

When the extra adrenal c the adrenal medulla concentration increases in th the lines of the presented expl action of corticoids whose cc is higher than that in the pe

APPEARANCE OF NEW CHROMAFFIN CELLS DUE TO CORTISONE ADMINISTRATION

The appearance of new chromaffin cells in the ganglia and the increase in the volume of the extra-adrenal chromaffin bodies can be explained by either one or both of two explanations

1 Cortisone might affect pre-existent but non-chromaffin cells in such a way that they begin to store catecholamines

2 Cortisone might bring about differentiation of chromaffin cells

It has been proved that in man the last stage of differentiation of chromaffin cells is the appearance of chromaffinity (Coupland 1952) According to Smitten (1963) this does not apply to all animal species In some species e g the mouse and chicken chromaffinity appears in the cells already at an early stage of morphological differentiation while in the rat only morphologically mature cells become chromaffin as was also observed in the present study

As was pointed out in Part I of the present study chromaffin cells are normally only occasionally present in the sympathetic ganglia of the rat at birth However cells with the morphological characteristics of chromaffin cells can be seen also in ganglia which are completely non-chromaffin The numbers of both of these cells gradually decrease with age

Since cortisone was found to increase the catecholamine concentration in mature chromaffin cells it is plausible that it would also be able to affect those pre-existent but still non-chromaffin cells so that they would begin to store catecholamines This would account for the increase in the number of chromaffin cells in the ganglia

In the extra-adrenal chromaffin bodies non-chromaffin cells are few if any The para-aortic chromaffin bodies are mature already at birth and they are homogenously chromaffin Therefore it would seem necessary to postulate that cortisone either stimulates proliferation of chromaffin cells in the para-aortic bodies or counteracts factors eventually inhibiting such proliferation

Mitotic figures are normally present in the chromaffin bodies at birth However in cortisone treated animals they are completely absent Thus no evidence is available that cortisone would cause an increased mitotic rate of chromaffin cells

The main bulk of the new chromaffin tissue which causes an increase in the volume of extra adrenal chromaffin tissue appears in the developing sympathetic ganglia in the immediate vicinity of the chromaffin bodies and so the increase in the amount of chrom affin tissue takes place in a manner from outside Indeed cells with nuclear characteristics of chromaffin cells are more nume rously seen in the ganglia just near the chromaffin bodies and if cortisone converts these non chromaffin cells to chromaffin it would be a reasonable partial explanation for the increase of chromaffin tissue

However the number of new chromaffin cells in the ganglia of cortisone treated animals markedly exceeds that of the non chrom affin but histologically similar cells in the ganglia of normal new born This suggests that cortisone is able not only to affect those pre chromaffin cells but also brings about cellular differentiation of new chromaffin cells

It is difficult to present any firm opinion about the develop mental stage and nature of these cells which appear to differentiate into chromaffin cells during cortisone administration However since late cortisone administration induced but a slight increase in the number of chromaffin cells in the sympathetic ganglia, the effect of cortisone clearly depends on the sufficiently early deve lopmental stage of the ganglia when primitive enough cells are still present in them

Most of the small dark and round cells which are numerous in the ganglia at birth and which continuously decrease in number with age are probably developing glial cells (mantle cells or satellite cells) The existence of primary undifferentiated cells in the ganglia at this stage of development is a question in dispute (for further discussion see Smitten 1963) However it is plausible that just these small dark cells are influenced by cortisone and differentiate into chromaffin cells when treated with it because except for chromaffin cells only developing ganglion cells and mature mantle cells exist in the ganglia

If it is tentatively accepted that cortisone causes differentiation of new chromaffin cells the question arises what is the mechanism which exerts this differentiation? The answer to this remains unclear as yet Although there is a voluminous previous literature concerning the effects of corticoids on the developing organism (*e.g* Karnofsky *et al* 1951 Sames & Leathem 1951 Fraser &

Fainstat 1951, Evans 1953 Fainstat 1954 Hublé 1957 Sobel 1958 Sobel & Freund 1958 Moscona & Karnofsky 1960 Fraser 1961, Whitehouse & Lash 1961 and others) there are no previous studies which would indicate that cortical hormones bring about primary cellular differentiation However there are evidences that corticoids especially glucocorticoids in some way accelerate the functional maturation of cells for ex the intestinal cells of the chick and the mouse and that the effect of corticoids depends on a sufficient differentiation stage of these cells (*e g*, Moog 1950 1952 1953 Moog *et al*, 1954 Moog Richardson 1955 Ross & Goldsmith 1955 Moog & Thomas 1955 1957)

Whether cortisone has an effect directly on developing cells or whether it indirectly influences an intermediate factor which then provides for differentiation of cells is unclear Cortisone has nevertheless an effect on the thyroid gland which is well known to influence cell differentiation (for references see Rapola 1962)

INTERMEDIATE EFFECT OF CORTICAL HORMONES ON THE CHROMAFFIN CELLS THROUGH THE THYROID GLAND

In the adrenal medulla cells now known to be the noradrenalin containing cells were shown to react to administration of thyroid hormone rather than the cells secreting mainly adrenalin (Eränkö 1958) Hopsu (1960) observed a marked increase in the amine concentration and in the volume of noradrenalin containing tissue in the adrenal medulla of thyroidectomized or thiouracil treated mice while feeding with thyroid powder caused a reverse effect

There is experimental and clinical evidence that administration of cortisone or ACTH suppresses thyroid function obviously inhibiting the thyroid stimulating action of TTH as measured by uptake of J^{131} at extrapituitary levels perhaps at the thyroid gland level (Epstein *et al* 1953)

It has been reported that the extra adrenal chromaffin tissue contains predominantly noradrenalin (see the Review of the Literature) Therefore also this tissue could be expected to react to changes in thyroid activity probably like the noradrenalin containing cells in the adrenal medulla If this were so cortisone besides through an eventual direct influence on the extra adrenal chromaffin cells might well do so by inhibiting the thyroid

In the present work it has for the first time been shown that cortical hormones possess a clear effect on chromaffin tissue

The question why the adrenal medulla has been enclosured by cortical tissue has often been discussed As a possible explanation it has been assumed that cortical hormones influence the methyl ation of noradrenalin (Shepherd & West 1951 Coupland 1953) However, one of the arguments in favour of this view i e that there is a direct relation between the cortex medulla ratio and percentage of adrenalin does not apply to all species (Eränko & Räisänen 1957)

The observations of the present study suggest that a sufficient level of cortical hormones is necessary for development and survival of the medullary chromaffin cells This would make enclosure of chromaffin cells inside the adrenal cortex a sensible protecting measure However in lower vertebrates cortical and medullary tissue forms separate glands The more primitive the phylogenetic form the greater the distance between the two components Apart from some teleosts (Baeker 1928) it is not until in the amphibians that the two adrenal components are in close contact with each other In phylogenetic series the two components approach each other, unite and become arranged On the other hand during ontogeny the adrenal glands of all mammals pass through the same stages that are final in lower species (Bachmann 1954)

As has been discussed previously in the present paper there are certain species dependent differences in the postnatal fate of the extra adrenal chromaffin tissue While this tissue is rather perma nent in some species *(e g* the guinea pig) it disappears during the early prepubertal phase in others *(e g* the rat and the mouse) However these findings do not invalidate the hypothesis that the cortical hormones possess a specific supporting effect on the chromaffin cells

The rat is a striking example of all animals in which hydrocor tisone has not been conclusively demonstrated either in the gland or in the blood even after intensive treatment with ACTH (Bush 1953 Endroczi & Yang 1960) As already discussed there is also a marked postnatal diminution in the size of the adrenal cortex of the rat These two things may be sufficient to explain the rapid dis appearance of extra adrenal chromaffin tissue in the rat

SUMMARY

Although it is generally accepted that in other mammals hitherto studied chromaffin tissue can be found outside the adrenals the rat has been thought to be an exception The existence of extra adrenal chromaffin tissue in the rat is therefore a question in dispute My own preliminary studies showed the chromaffin tissue of the rat to be of unusual interest and a comprehensive study was carried out

The material consisted of rat fetuses and young rats aged from 16 days after copulation to 60 days after birth Adult animals were also studied After fixation usually in dichromate formalin serial sections were prepared of the retroperitoneal tissue block from the upper end of the adrenals to the lower end of the kidneys In addition to unstained sections studied for the chromaffin reaction Schmorl s ferro ferricyanide technique was used For histological studies fixation was carried out in Bodian s fixing fluid and the sections were stained with haematoxylin eosin van Gieson haemat oxylin orcein picroindigocarmin or with Bodian s Protargol impreg nation method The volumes of both intra and extra adrenal chromaffin tissue were planimetrically determined from serial dichromate formalin fixed frozen sections

The following main results were obtained

I NORMAL PERINATAL CHANGES IN THE CHROMAFFIN TISSUES

Extra adrenal chromaffin bodies In rat fetuses aged 16—17 days after copulation well defined extra adrenal chromaffin bodies were observed in the vicinity of the abdominal sympathetic ganglia The main body the organ of Zuckerkandl was on the aorta below the origin of the superior mesenteric artery Smaller encapsulated chromaffin bodies were found near the developing suprarenal ganglia and the coeliac ganglion

The intensity of the chromaffin reaction in the para aortic bodies increased with age and reached its maximum at the time of birth when almost all the cells in the bodies were chromaffin

During the first week after birth the volume of the extra adrenal chromaffin tissue decreased because of cell degeneration Degenerative changes included a loss of chromaffinity, nuclear irregularity and pyknosis and vacuolization of the cytoplasm Two weeks after birth only a few faintly chromaffin cells were found in the degenerating tissue The main body usually disintegrated during the third or fourth week after birth

Adrenal medulla Migration of future medullary cells from the nearby sympathetic ganglia began around the 16th day after copulation and continued until birth A faint chromaffin reaction was observed in them 20—21 days after copulation In newborn rats the reaction was still weaker than that in the extra adrenal chromaffin bodies After birth the intensity of the reaction incre in the medullary cells and the medulla became distinctly se from the cortical tissue in a few days

Sympathetic ganglia At the time of birth the ganglia were very immature In addition to develop cells numerous small basophilic polynucleolar cells The number of these cells gradually diminished wit were only occasionally found after the first postnat chromaffin cells were also found in the ganglia After a few days they were rarely found

II EFFECT OF CORTICAL HORMONES AND ACTH ON THE CHROMAFFIN CELLS

Administration of cortisone or hydrocortisone not only prevented the postnatal disappearance of the extra-adrenal chromaffin tissue but brought about the appearance of new chromaffin cells in the extra-adrenal chromaffin tissue — thus increasing its volume — and also in the sympathetic ganglia. At the same time the intensity of the chromaffin reaction increased in individual chromaffin cells.

The effect was dependent on the dosage. Small doses only prevented the normal involution of extra-adrenal chromaffin tissue while larger doses produced a progressive increase in its volume. With the same daily dosage most marked changes were obtained when the hormone treatment was begun immediately after birth. The later the administration was initiated the smaller was the increase in the amount of extra-adrenal chromaffin tissue. Treatment begun 15 days after birth had no clear effect. On the other hand hyperplasia of the extra-adrenal chromaffin tissue brought about by treatment with cortisone remained unchanged for at least 50 days after discontinued treatment.

Large doses of ACTH prevented the disappearance of the extra-adrenal chromaffin tissue but did not cause the appearance of new chromaffin cells. Even large doses of desoxycorticosterone were without any effect on the extra-adrenal chromaffin tissue.

Cortical hormones and ACTH had no clear effect on the volume or chromaffin reaction of the adrenal medulla.

The mechanism and significance of the effect of cortical hormones on chromaffin cells are discussed. It is tentatively concluded that cortisone or hydrocortisone may stimulate the differentiation of new chromaffin cells and/or the maturation of pre-existing phaeochromoblasts into chromaffin cells.

It is suggested that the normal postnatal degeneration of extra-adrenal chromaffin bodies of the rat may be due to a decrease in the level of cortical hormones associated with birth. Chromaffin cells of the adrenal medulla enclosed within cortical tissue may be saved from degeneration by the presence of sufficient local concentration of cortical hormones. If this is so it would provide a teleological explanation for the close association in mammals of the cortical and medullary tissues of the adrenal gland.

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EVIDENCE FOR THE EXISTENCE OF MONOAMINE-CONTAINING NEURONS IN THE CENTRAL NERVOUS SYSTEM

I Demonstration of Monoamines in the Cell Bodies of Brain Stem Neurons

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FROM THE DEPARTMENT OF HISTOLOGY KAROLINSKA INSTITUTET STOCKHOLM, SWEDEN

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INTRODUCTION

By means of a sensitive fluorescence method it has been shown that catecholamines and 5 HT[1] in the mammalian central nervous system are accumulated in very high concentrations in synaptic terminals belonging to special systems of neurons which are probably monoaminergic (CARLSSON FALCK and HILLARP 1962 CARLSSON FALCK FUXE and HILLARP 1964 DAHLSTROM and FUXE 1964*a* FUXE 1964*a*) In the present and following papers of this series it will be shown that there exist two distinctly different types of neurons which contain in their cell bodies low concentrations of a primary catecholamine (DA or NA) and 5 HT respectively Evidence will also be presented for the view that these neurons are monoaminergic and give rise to monoamine containing synaptic terminals

[1] *Abbreviations used* DA = dopamine NA = noradrenaline A = adrenaline 5 HT = 5 hydroxytryptamine 5 HTP = 5 hydroxytryptophan dopa = 3 4 dihydroxyphenyl alanine MAO = monoamine oxidase

MATERIAL AND METHODS

The brain stem was examined in about 400 male albino rats (200–250 g body wt about 2 months old) About 300 of the animals were treated in various ways (see *Table I*) with drugs interfering with the monoamine metabolism Untreated animals were used in all such experiments as controls the pieces of brain stem from both the experimental and control rats being removed and treated in exactly the same way

The brain stem from a number of mice and guinea pigs and from a few rabbits and cats was also examined

Reserpine (Serpasil) nialamide (Niamid) iproniazide (Marsilid) and MO 911 (Pargyline hydrochloride) were kindly donated by Swedish Ciba (Stockholm) Swedish Pfizer (Stockholm) Hoffmann La Roche Ltd (Basle) and Abbott Laboratories (Chicago Ill) respectively The other substances used were obtained from the Regis Chemical Company (DL α methyl *m* tyrosine DL-5 hydroxytryptophan) and the Sigma Chemical Company (DL-*m* tyrosine L-dopa) All substances with the exception of reserpine were *administered immediately after preparation of the solutions* Iproniazide MO 911 *m* tyrosine α methyl *m* tyrosine and 5 HTP were dissolved in 0 9 % NaCl the amino acids under gentle warming (+50 C to +60 C) L-dopa (13 mg/ml) and nialamide (20 mg/ml) were dissolved in 0 9 % NaCl with the help of a minimal amount of dilute HCl and gentle warming Polyethyleneglycol (0 02 ml/ml) was then added to the warm solution and pH carefully brought to about 5 with *N* NaOH The solvents for the drugs were usually not given to the control animals since such a procedure has been found both in this and Dr Falck s laboratory to have no demonstrable effect

The rats were killed by decapitation under light ether anesthesia The brain stem was then immediately taken out dissected and frozen etc identically for all animals In some animals the brain stem was dissected out in the living animal which was kept under nembutal anesthesia (1 ml of a 1 % solution) and immediately frozen No certain differences in the fluorescence microscopical picture could be observed however as compared with the animals killed in the manner described above The brain stem was divided into 4 pieces which consisted of the dience

TABLE 1 *Time before killing the animals after the first injection*

Number of animals within parentheses

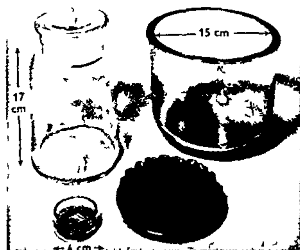
Drug	Dose mg/kg body wt	Hours
serpine i p	10	½ (6) 1 (7) 2 (7) 4 (8) 6 (5) 8 (8) 12 (6) 24 (10) 48 (6) 72 (6) 9
	1	4 (6) 24 (6)
	0 1	4 (6) 24 (6)
	0 05	4 (6)
alamide i p	500	5–6 (50)
serpine i p + }	10	24
alamide i p }	500	5–6 (25)
roniazide i v	200	17 (8)
O-911 i p	300	1 (5) 2 (5) 3 (5) 4–5 (8)
	400	3–5 (8)
alamide i p + }	500	5–6
dopa s c }	100	½ (6) 1 (10)
alamide i p + }	500	5–6
L 5 HTP s c }	75	1 (6)
Tyrosine i p	400	6 (12) (3 doses with 2 h intervals)
Methyl *m* tyrosine i p	400	24 (12)
	400	9 (6) 12 (6) 15 (6) 18 (6) 24 (10) (2 doses with a 2 h interval)

phalon mesencephalon pons and medulla oblongata The specimens were placed on small pieces of paper with a code number for identification The pieces were rapidly frozen in liquid propane (Gasolpropan O K b p −41 C) cooled by liquid nitrogen The specimens were then transferred to a pig made out of lead and tin also cooled by liquid nitrogen and covered with metal net cylinders 1 cm high and with diameters ranging from 1 5–4 cm (Text fig 1) The specimens had a good contact with the pig and did not cover each other When all the specimens had been transferred to the pig the latter was placed in a glass jar (Text fig 1) belonging to the freeze drying apparatus The glass jar had previously been placed in a bath of ethyl alcohol cooled to −45 C by a cooling coil from a cooling apparatus of compressor type The temperature of the bath could be easily regulated by a contact thermometer The glass jar with the pig was rapidly adhered to the freeze drying apparatus The specimens were freeze dried for 7 days with liquid nitrogen in the cold trap which was about 4 cm away from the specimens The pumps used were a gas ballast rotary high vacuum pump model 2SC20A and an

oil vapour diffusion pump model 102 A Edwards High Vacuum Ltd England The bath of ethyl aclohol surrounding the jar with the specimens was kept for the first five days at a temperature of −35°C At the end of the fifth day the temperature was raised to −20 C At the end of the sixth day the bath was exchanged for water of about +35 C Early the seventh day this water was exchanged for hot water (+50 C) to ensure that the tissues had at least reached room temperature before the pumps were turned off one hour later No second cold trap for water was used Instead the oil in the pumps was changed when necessary

Immediately after the pig with the specimens had been taken out of the freeze drying apparatus it was transferred to an exicator containing phosphopentoxide (Sicapent Theodor Schuchardt GmbH Munich) The specimens were then carefully transferred to nylon nets (see Text fig 1) in another exicator They were kept here for a maximum of 2–3 hours To obtain constant results it was important to perform the freeze drying procedure in the same way every time since the water contents of the tissues greatly influence the histochemical reaction

Not only the freeze drying but also the histochemical procedure has to be standardized however The latter procedure like the former follows to a large extent that described by FALCK (1962) but has now been standardized to obtain reproducible results with the central nervous system It cannot be used on other tissues without previous trials Standardization has been made possible mainly by the use of paraformaldehyde (purum E Merck Darmstadt Germany) with a constant water content The paraformaldehyde was dried by storage for 7–10 days in exicators containing phosphopentoxide It has by then lost so much of its adsorbed water that no fluorescent structures developed in the brain stem when the latter was treated with it for 1 h at +80 C Paraformaldehyde has been stored in this laboratory over solutions of sulphuric acid with different densities for 5–7 days at a temperature of +20–22 C (HAMBERGER and MALMFORS 1964) If paraformaldehyde was used that had been stored over a solution giving a low relative air humidity (10–30%) there was obtained no or a distinct but weak reaction If on the other hand there was used paraformaldehyde stored over a solution giving a high relative air humidity (85–95%) a strong reaction was obtained but the fluorescent structures were often diffuse The best results were obtained when the paraformaldehyde had been stored over a solution having a density of about 1 33–1 25 giving a relative air humidity of 50–70% 10 g of the paraformaldehyde thus obtained was put into glass jars (see Text fig 1) having a volume of 1 liter One nylon net with 20–25 tissue



TEXT FIG 1 The pig with the metal net cylinders is seen to the right This pig fits into the glass jar (also on right) that belongs to the freeze drying apparatus The nylon net on which the specimens are placed after freeze drying is seen to the left stretched out between two plastic rings This net is then transferred to the glass jar (left of the figure) in which the histochemical reaction is performed

specimens on it was put into each jar which was tightly closed with the use of high vacuum grease The glass jars were then placed for 1 h in an oven at a temperature of +80 C Some pieces from the different parts of the brain stem were also treated for 3 h The tissue specimens were kept after the histochemical treatment in an exsiccator for a maximum of 24 h after which they were embedded in paraffin *in vacuo* (Merck m p −51–53 C) for 5 min at +60 C The sections were made 8–10 μ thick and stretched on the slides by heating to +55–65 C They were then allowed to cool after which they were mounted in Entellan (Merck) to which a small amount (0 04 ml/10 ml Entellan) of xylene had been added By heating the slides again it was possible to dissolve enough of the paraffin for the amount remaining around the section not to disturb the fluorescent picture The mounted sections must be stored in darkness since the fluorescent products are sensitive to light The slides can be stored mounted for at least 5–7 days without any noticeable decrease in fluorescence intensity Sections which have not been deparaffinized however can be stored in a dry place for at least 2–3 weeks without any change in fluorescence picture Tissues in paraffin blocks can be stored in a dark and dry place for at least 2–3 months

Fluorescence microscopy was performed with a Zeiss monocular fluorescence microscope with an HBO 200 high pressure mercury lamp (Osram Germany) fitted with a BG 12 filter (3 or 4 mm Schott & Gen Germany) and 2 KG 1 filters (1 mm Schott & Gen. Germany) There was used a dark field condensor for immersion oil (Zeiss) and the lenses were planeachromates The secondary filter was a Zeiss 50 filter (2 mm) which corresponds roughly to an OG 4 filter (3 mm Schott & Gen Germany) Microphotographs were taken with Scopix G film (Gevaert) Exposure times ranged from 20–90 sec

Since it has been found (see Results) that especially the fluorescent product formed from 5-HT is highly sensitive to ultraviolet light great care must be taken not to expose the sections to this light unnecessarily

The peak of maximum fluorescence activation was examined in a microscope fitted with a high pressure xenon lamp (Osram XBO 1600 W) The light was passed through a water cooled 1 mm Schott BG 38 filter (for heat absorption) and then through a monochromator (Zeiss M4Q III) The exit slit was projected in the diaphragm of the condensor (light or dark field) of a fluorescence microscope (Zeiss) with a Wratten 15 filter in the tube

Transverse sagittal and horizontal sections were made The transverse sections through the pons and medulla oblongata were cut as perpendicular as possible to the longitudinal axis of the brain stem Transverse sections through the mesencephalon were made following the axis given in the book by König and Klippel (1963) approximately corresponding to the A–A1 axis in the book of Zeman and Innes (1963) Transverse sections through the mesencephalon were also made according to the B–B1 axis also given in the latter book Every fifth section was collected. After they had been examined and photographed in the fluorescence microscope a large number of sections from the medulla oblongata pons mesencephalon and diencephalon were fixed in Serra s solution (1946) and then stained with toluidine blue or Einarsson s chromalum gallocyanin (Einarson and Bentsen 1939) The sections for toluidine blue staining were thoroughly washed in distilled water of a pH of 6 0 before staining in a 0 5 % toluidine blue water solution of the same pH for 30–60 seconds The slides were then quickly rinsed in distilled water of pH 6 0 differentiated in 96 % ethyl alcohol and finally mounted in Entellan (Merck) These techniques—especially that of Einarson—were found to give a good Nissl picture The staining due to ribonucleic acids was examined by digestion with ribonuclease (Brachet 1953) To permit a more accurate study of the Nissl substance the brain stem was fixed by perfusion with 10 % neutral formaline

for 10 minutes a m BODIAN and TAYLOR (1963) The fixation of the specimens was continued for 12–24 hours in 10 % neutral formaline before embedding in paraffine This avoids the freeze artefacts often observed in the cell bodies in freeze dried sections The slides were stained in EINARSON's chromalum gallocyanin or in toluidine blue as described above except that they were stained for 5–10 minutes

The intensity of the fluorescence of the cell bodies was classified as very weak weak medium or strong

For the medulla oblongata and pons the terminology used is that of ZEMAN and INNES (1963) For the reticular and raphe nuclei in these areas see VALVERDE (1961) For the mesencephalon and diencephalon the terminology is that of KONIG and KLIPPEL (1963)

I Specific fluorescence in nerve cells

Nerve cells belonging to several nuclei in the brain stem—but no cells of other kinds—have been found to develop fluorescence on formaldehyde treatment There is very good evidence (see below) that this fluorescence is specific that is to say is due to the presence of any of the monoamines which can be converted to intensely fluorescent products under the conditions of the histochemical method The fluorescence is associated to the cytoplasm of the cell bodies and often also to the larger processes (Figs 1 4 17) the cell nuclei being non fluorescent unless diffusion has occurred With perfect freeze drying the fluorescence could be seen to have a character so diffuse that the fluorescent compounds must be conceived as either diffusely present in the cytoplasm or bound to structures—such as the monoamine storage granules—with a widespread distribution within the cells Freeze artefacts however often arose in the nerve cells making the fluorescence more or less uneven Many nerve cells of the catecholamine type especially those within groups A5 A6 and A7 showed an accumulation of the fluorescent compounds around the nucleus (Figs 15 16 18)

The brain stems treated with formaldehyde gas for 3 h differed from those treated for only 1 h solely by an increased background fluorescence The 5 HT type cells (see below) had the same appearance in all normal animals independently of whether the brain stem specimens had been removed after killing (decapitation) or *in vivo* (nembutal narcosis) and immediately been frozen

The nerve cells were found to be of two distinctly different types

1 Nerve cells which show a weak to strong green fluorescence in normal animals and animals treated with MAO inhibitors The light emitted may be more yellow green if the formaldehyde treatment is prolonged to 3 h

2 Nerve cells which in untreated animals show a very weak or weak yellow fluorescence or none at all but a medium or usually strong yellow fluorescence after the administration of potent MAO inhibitors The strong fluorescence arising in these cells after MAO inhibition often does not appear in the microphotographs however since the fluorescence rapidly decreases in intensity on UV irradiation (see below)

There is good evidence (see below) *that the fluorescence of the two cell types is due to the presence of primary catecholamines and 5 HT and they will therefore be referred to in the following as nerve cells of the catecholamine and 5 HT type respectively*

The monoamine containing nerve cells have a widespread distribution within the lower brain stem It is at present difficult to systematize them on account of our scanty knowledge of what functional or anatomical systems they belong to The best known cells are a large group in the substantia nigra which give rise to a fibre tract ending with DA containing terminals in the caudate nucleus and putamen (ANDÉN CARLSSON DAHLSTRÖM FUXE HILLARP and LARSSON 1964) and smaller groups of cells in the medulla oblongata sending axons down into the spinal cord to end with NA and 5 HT terminals in the gray matter (CARLSSON FALCK FUXE and HILLARP 1964 DAHLSTROM and FUXE 1964*b*) Where possible however the cells have been divided into groups This has been done with consideration not only to the topographical conditions and to the morphology of the cells but also with a view to the degree to which the cells seem to form more or less continuous systems separated from other cell systems It is possible—and perhaps even probable—that the division is partly artificiel This is true especially of the 5 HT type cells A division of some kind or other is necessary however for practical reasons

For the sake of completion there have been described also a few small groups of catecholamine cells in the hypothalamus and posterior thalamus No other groups of monoamine containing cells have been found in the brain stem Nor have such cells been observed in the spinal cord cerebellum or cerebral cortex but it must be pointed out that no systematic study of the cerebral cortex has as yet been made *It therefore seems probable that most of the numerous systems of monoamine terminals present in the different parts of the brain and spinal cord derive from axons belonging to the special neuron groups in the lower brain stem* This is currently being studied by means of transections of the brain stem at various levels

II Localization and morphology of monoamine containing nerve cells

1 MEDULLA OBLONGATA

Nerve cells of the catecholamine type

GROUP A1 This group is of medium size and consists of a bilateral horizontal band of numerous small to medium sized nerve cells of weak to me

dium fluorescence which were seen to form a connected system from the level of the motor decussation up to that of the cranial third of the inferior olivary complex In the caudal part the cells were situated immediately lateral to the nuc reticularis lateralis lying fairly close together At the caudal third of the inferior olivary complex they began to separate lying laterally ventrally, and medially of the nuc reticularis lateralis This localization was maintained also in the rostral part of the cell group In a transverse section most cells were observed at the mid olivary level The cells were oval or spindle shaped (Fig 2) and many were clearly multipolar (Fig 1) In the caudal part the cells had a somewhat stronger fluorescence intensity than in the rostral part The nerve cells had fine to coarse moderately stained Nissl granules, while the large non fluorescent multipolar cells of the nuc reticularis lateralis contained large coarse deeply stained Nissl bodies There is good evidence that most of the cells in this group have axons that form descending fibres in the spinal cord and which end with NA containing synaptic terminals in the gray matter (DAHLSTROM and FUXE 1964b)

GROUP A2 A small group of small to medium sized round to oval nerve cells were observed together with green fluorescent varicose fibres in the closed portion of the medulla oblongata in the area where the nuc tractus solitarii nuc motorius dorsalis n vagi and nuc commisuralis are situated (Fig 3) Most of the cells were localized in the ventrolateral part of nuc commisuralis Some cells were also present just lateral to the nuc dorsalis motorius n vagi The cells had a weak or medium fluorescence and sometimes showed fluorescent processes (Fig 4) The cell bodies contained moderately stained fine to coarse Nissl granules Ventral to these cells were present the non fluorescent medium sized cells of the nuc motorius dorsalis n vagi with large darkly stained Nissl bodies especially close to the cell membrane Still more ventrally were seen the large multipolar cell bodies of the nuc n hypoglossi with large deeply stained Nissl granules A few medium sized oval cells of a medium fluorescence were observed in the rostral parts of the nuc tractus solitarii

GROUP A3 A few small very weakly to weakly fluorescent oval cells were observed in transverse sections in the nuc olivaris accessorius dorsalis

GROUP A4 At the level of the maximum development of the nuc n facialis were seen in transverse sections a row of medium sized round to oval cell bodies of medium to strong fluorescence in the lateral part of the roof of the fourth ventricle just under the ependyma ventral to the cerebellar nuclei (Fig 5)

Nerve cells of the 5 HT type

Since the yellow fluorescence observed in the nerve cells is—unlike the green—very weak to weak or sometimes absent the following description will be based mainly on data from nialamide treated animals There is good evidence (see below) that the administration of a potent MAO inhibitor causes such a high accumulation of 5 HT in these cells that the cell bodies show a medium or usually a strong yellow fluorescence and that also their processes—or at least the larger ones—become clearly visible Often however this strong fluorescence could not be demonstrated in the micro photographs since the intensity of the fluorescence rapidly declined on exposure to ultraviolet light (see below)

The localization of the nerve cells of the 5 HT type is entirely different from that of the green fluorescent nerve cells They are thus found pre dominantly in the raphe region and this holds true to a large extent also of their localization in the pons and mesencephalon while the green fluorescent cells in the lower brain stem occupy a more lateral position except in the cranial part of the mesencephalon

Group B1 This group is of medium size and its small to medium sized cells surrounded the medial and ventral surface of the pyramidal tract from the level of the motor decussation up to the beginning of the nuc n facialis Most of the cells were present within the nuc raphe pallidus (Fig 7 10) It may well be that this group consists in reality of at least two group one comprising the cells within nuc raphe pallidus and the other group the rest No such division was made however since these cell groups could not be clearly separated from each other Most—maybe all—of the cells form fibres that pass down into the spinal cord and which end with 5 HT containing terminals in the gray matter (Carlsson Falck Fuxe and Hillarp 1964 Dahlstrom and Fuxe 1964b)

At the level of the decussatio pyramidum many cells were seen out lining the medial surface of the pyramidal tract (Fig 6) B1 cells were also present at the emergence of the radices n hypoglossi and ventral to the nuc reticularis lateralis just under the ventral surface of the brain (Fig 8) The small cells had a minimal amount of cytoplasm containing fine lightly stained Nissl granules while the medium sized cell bodies contained coarse to fine moderately stained Nissl granules Fine green fluorescent varicose fibres were seen at the level of the inferior olivary complex lying in close contact with the yellow fluorescent cells in the area of the nuc raphe pal lidus (Fig 7) and at the emergence of the radices n hypoglossi

Group B2 This is a well defined small group of small to medium sized cells present within the area of the nuc raphe obscurus (Fig 9) The cells

were round to oval and the long axis of the cell bodies was often parallel to the raphe The Nissl picture was the same as for group B1 The axons from many—maybe all—of the cells were seen in horizontal and sagittal sections to run towards the spinal cord close to the raphe Just below the level of the motor decussation these axons had joined the axons from group B1 and were lying fairly close to the ventral surface of the brain lateral to the pyramidal tract

GROUP B3 This group is fairly large The cells surrounded the pyramidal tract at the different levels of the nuc n facialis At the caudal level however B1 cells were still present on the medial side of the pyramidal tract (Fig 7) within nuc raphe pallidus It must be pointed out however that no distinct borderline existed between group B1 and B3 The cells of the B3 group were present mainly among the fibres of the lemniscus medialis just dorsal to the pyramidal tract (Figs 10 12) and within an area just lateral to the pyramidal tract (Fig 11) corresponding to the nuc paragigantocellularis lateralis in the cat (TABER 1961) A number of cells were present within nuc raphe magnus and a number of small cells were found ventrally and medially of the pyramidal tract Cranially many cells were present in the lower portion of the pons just dorsal to the trapezoid body still mainly lying among the fibres of lemniscus medialis (Fig 21) At this level only a few cells were observed within nuc rapho magnus and a few small cells ventrolaterally of the trapezoid body By the level of the cranial part of the nuc motorius n trigemini the cells had disappeared The cells were mainly medium sized round to oval or sometimes spindle shaped and many were clearly multipolar (Figs 11 12) The fluorescent processes and the long axis of their cell bodies often followed the contour of the tractus pyramidalis and the trapezoid body They contained a coarse intensely stained Nissl substance as did—to a much higher degree—the large cells of the nuc n facialis Many of the B3 cells especially those in the caudal part seemed to send axons down towards the spinal cord mainly lying just lateral to the pyramidal tract

GROUP B4 In transverse sections a few small to medium sized round to oval shaped cells were also observed just under the fourth ventricle dorsal to the vestibular nuclei and nuc nervi abducens

Area postrema Many small nerve cells of both the catecholamine and 5 HT type were present in the area postrema the former especially in the dorsal part the latter in the ventral part The 5 HT type cells became clearly visible only after treatment with potent MAO inhibitors A detailed study on the area postrema will be reported in a separate paper (FUXE and OWMAN 1964)

Nerve cells of the catecholamine type

GROUP A5 A small group of medium to strongly fluorescent medium sized oval cells which often were seen to be multipolar were present among the fibres of the tractus rubro spinalis mainly at the level of the caudal and middle third of the nuc olivaris superior medial to the outgoing fibres of the n facialis and lateral to the nuc olivaris superior (Figs 13 14) Some of these cells were also present dorsal to the nuc olivaris superior and a few also along the medial side of the n facialis dorsal to the tractus rubro spinalis The cells were still seen in the tractus rubro spinalis at the level of the nuc motorius n trigemini but were now situated medial and ventral to the radix n trigemini The cells had coarse intensely stained Nissl granules The green fluorescence in many of the cells was not equally distributed but was present especially in the zone surrounding the nucleus The processes—presumably axons—of some cells were seen to run dorsally parallel to the fibres of the facial nerve probably *via* to the nuc motorius n trigemini These axons had a distinct green fluorescence and developed varicosities at a short distance from the pericarya

GROUP A6 This group seems to be identical with the locus coeruleus and is quite unique as regards its cell composition All—or at least practically all—of its closely packed nerve cells (Figs 15 19) belong to the catecholamine type The cells all present the same general appearance and show a strong green fluorescence especially in an often conspicuous zone around the nucleus (Figs 16–18) In some of the cells this zone showed a dusty appearance (Fig 18) In the periphery of the nucleus where the cells are not so closely packed (Figs 16–17) the larger processes of the multipolar cells can be easily seen due to their fairly strong fluorescence Some of these processes which presumably were axons and had the same appearance as those of the A5 group were seen to run medially to the raphe Others seemed to run ventrally to the nuc motorius n trigemini At the level of the caudal third of the griseum pontis the locus coeruleus had been reduced to a few small fluorescent cells Instead there began to appear numerous fine green fluorescent varicose fibres The cell were medium sized and had coarse intensely stained Nissl granules in contrast to the large non fluorescent cells of the nuc tractus mesencephalici n trigemini which contained fine evenly distributed Nissl granules

A row of strongly fluorescent medium sized multipolar cells (Fig 19) with the same general appearance as the locus coeruleus cells has been observed to pass from the ventral part of the rostral portion of the locus

coeruleus in an arch medial to the nuc. motorius n. trigemini down to the cells within group A5.

GROUP A7. At the level of the caudal third of the griseum ponti, a small group of medium sized to large, oval cells of strong green fluorescence was present within the formatio reticularis ventral to the ventral portion of the pedunculus cerebellaris superior dorsal to the tractus rubro spinalis (Fig. 20). These cells had often a strong green fluorescent zone around the nucleus.

Nerve cells of the 5-HT type

GROUP B3. This group has already been described. See above.

GROUP B5. At the level of the nuc. motorius n. trigemini a number of small to medium sized, round to oval and sometimes clearly multipolar cells were localized within the nuc. raphe ponti. The axis of the cell bodies was often parallel to the raphe.

GROUP B6. A small group of cells which have the same general appearance as but are more numerous than those of group B4 was present in the middle line just under the fourth ventricle, especially its cranial part (Fig. 22). The cells of groups B5 and B6 had fine to coarse, moderately stained Nissl granules. By the level of the caudal third of the griseum ponti, the cells of groups B5 and B6 had increased in number.

MESENCEPHALON

The caudal part of the mesencephalon contains almost exclusively nerve cells of the 5-HT type, while the monoamine containing cells in the rostral part are almost exclusively of the catecholamine type. The number of 5-HT cells is at least as great as that of the 5-HT cells in the pons and medulla oblongata together, while the catecholamine cells form a huge collection that far outnumbers the corresponding cells in all other parts of the brain stem together. This great collection can be divided topographically into three groups: cells in the reticular formation (A8), in the substantia nigra area (A9) and in the basal medial area mainly dorsal to the interpeduncular nucleus (A10). It must be pointed out that there is no distinct border line medially between A9 and A10 and laterally between A9 and A8 (Text fig. 4 and 5). Nor could any distinct border line be seen between the ventrolateral part of A10 and the ventromedial part of group A8 (Text fig. 3).

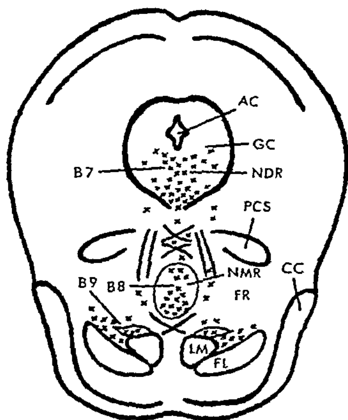
Nerve cells of the catecholamine type

GROUP A8. This large group of medium green fluorescent cells was present within the reticular formation (Text figs. 3-4). The cells of this group were mainly concentrated within a transverse plane just behind the nuc.

ruber together with fine varicose intensely green fluorescent fibres Here they were mainly present dorsal to the lateral part of the lemniscus medialis (Fig 23) but could also be seen scattered over the part of the reticular formation situated dorsal to the medial part of the lemniscus medialis (Text fig 3) With the appearance at a somewhat higher level of the nuc ruber the cells were pushed aside and confined to the lateral part of the reticular formation and the ventral part ventral to the nuc ruber (Text fig 4) No distinct borderline was observed ventromedially against group A10 (Text fig 4) or ventrolaterally against group A9 (Text fig 4) As the nuc ruber grew larger the cells of this group gradually disappeared (Text fig 5) The cells were mainly of medium size but some were small They were round to oval and the medium sized cells were often clearly multipolar (Figs 24 25) The medium sized cells had coarse intensely stained Nissl granules while the small cells had only fine moderately stained Nissl granules Medial and ventral to the cells were seen the large multipolar cells of the nuc ruber with coarse deeply stained Nissl bodies

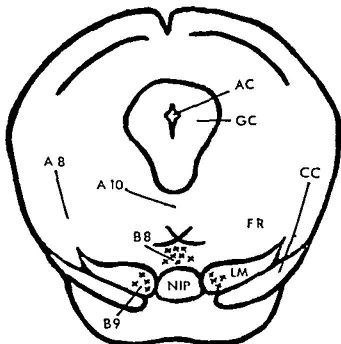
GROUP A9 This large group of medium fluorescent cells was present mainly within the substantia nigra (Text figs 4 5 and 6 Fig 26) Most of the cells were present within the zona compacta (Figs 27 28) of the substantia nigra but several also within the zona reticulata (Fig 26) and a few also within the pars lateralis Cells of the catecholamine type have been found within the substantia nigra also in the cat and have been found to be most numerous in the zona compacta This has been verified also for *Cynomolgus* monkeys (DAHLSTRÖM and FUXE unpublished data) In the cranial part of this group many cells were present also within the area ventralis tegmenti[1] (Text fig 6 Figs 30 31) They were here found to surround the nonfluorescent nuc tractus opticus basalis with its tractus (Fig 32) However no distinct border line was observed between zona compacta and area ventralis tegmenti and it was difficult to decide whether some of the green fluorescent cells belonged to the zona compacta or to the area ventralis tegmenti The cell group ended at approximately the same level as the zona compacta disappeared They were at this level strongly reduced in number and present mainly within the zona compacta but probably also within the most caudal part of the zona incerta Group A9 was medially not clearly separated from group A10 (Text figs 4 5 Fig 29) The border line zone between these groups was present mainly in the area[1] medial to the zona compacta and lateral to the nuc interpeduncularis which has

[1] However most of the fluorescent cells within these areas should preferrably be referred to group A10 since they probably are not a part of the nigro neostriatal system but presumably of the limbic system (see discussion page 47)



TEXT FIG ‥ Transverse section through the mesencephalon at a level just behind the nuc interpeduncularis. The frontal planes are cut with the axis of KONIG and KLIPPEL. The axis i used also in Text figs. 3–6 The topography of groups B7 B9 is illustrated and the 5-HT type cell are indicated as crosses AC Aqueductus cerebri FL Fasciculus longitudinalis; FP Formatio reticularis GC Grisea centralis LM Lemniscu medialis; NDP Nuc dorsalis raphe NMP Nuc medianus raphe CC Crus cerebri, PCS Pedunculus cerebellaris superior

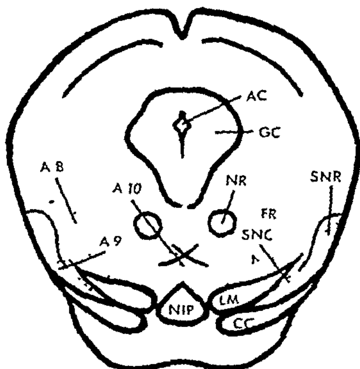
been found to contain numerous cells of the catecholamine type Laterally group A9 was not clearly separated from A8 (Text fig 4 Fig 23) The cells were of the same type as group A8 and had the same Nissl picture as thi group Group A9 and A10 give rise to fibre bundles which run cranially mainly within Forel s field H_ and within the ventral portion of the cru cerebri The part of the fibres within the crus cerebri was seen mainly to pass into the capsula interna and to send fibres up towards the nuc caudatu putamen At least most of the fibre bundles within Forel s field H_ and a number of bundles also within the crus cerebri were seen to pass into the lateral part of the hypothalamus lying mainly within fasciculus



TEXT FIG 3 Transverse section through the mesencephalon at the level of the beginning of the nuc interpeduncularis The topography of groups A8 and A10 and of groups B8–B9 is illustrated The catecholamine type cells are indicated with dots and the 5 HT type with crosses *NIP* Nuc interpeduncularis For the other abbreviations see Text fig 2

medialis prosencephalici some of them probably ending with NA containing terminals in the hypothalamus In fact it was possible in sagittal and horizontal sections to see how fibres arose from the bundles to terminate within the different nuclei of the hypothalamus The destination of the other fibres is at the present time unknown It has been shown experimentally with the help of lesions (ANDÉN CARLSSON DAHLSTRÖM FUXE HILLARP and LARSSON 1964) that group A9 mainly sends fibres via crus cerebri and capsula interna to the neo striatum Consequently group A10 probably mainly gives rise to fibres situated within Forel s field H_2 and the fasciculus medialis mesencephalici some of which at least probably terminate within the hypothalamus

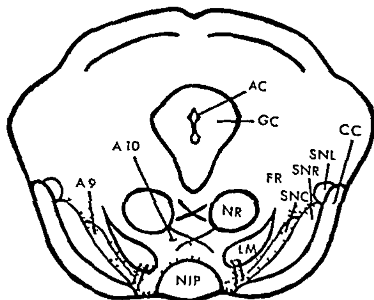
GROUP A10 This is the largest group in the mesencephalon (Text figs 3–5) Its medium fluorescent cells were mainly situated in the area dorsal to the nuc interpeduncularis (Fig 33) caudally within the caudal part of the nuc linearis and cranially mainly within an area which corresponds to



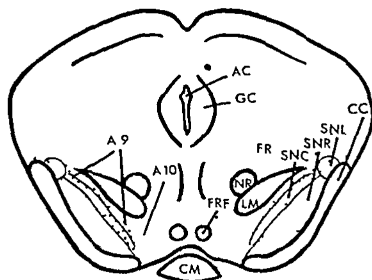
TEXT FIG 4 Transverse section through the mesencephalon at the level of the middle third of the nuc interpeduncularis The topography of groups A8–A10 is illustrated The catecholamine type cells are indicated with dots NR Nuc ruber SNC Substantia nigra zona compacta SNP Substantia nigra, zona reticulata For the other abbreviations see Text figs 2 and 3

the nuc parabrachealis pigmentosus in the cat (TABEI 1961) (Figs 34 35) Postally group A10 rapidly decreased after the nuc interpeduncularis had disappeared Some cells however were still present within the nuc interstitialis decussationis ventralis tegmenti and between the fascicula retroflexa just cranial to the nuc interpeduncularis The fluorescent cells just lateral to the nuc interpeduncularis are also referred to this group

A number of cells were also present within nuc linearis pars intermedialis and a few within nuc n oculomotorii and nuc Edinger Westphal As stated above this group is not clearly separated from group A8 and A9 (Text fig 4 and 5 Figs 29 33) The cells of group A10 were small to medium sized but the small cells were in the majority The cells had the same Nissl picture as groups A8 and A9 As already mentioned group A10 probably mainly sends its fibres cranially within Forel s field H_2 and the fasciculus medialis prosencephalici some of them—at least probably—terminating in the hypothalamus



Text fig 5 Transverse section through the mesencephalon at the level of the cranial third of the nuc interpeduncularis The topography of groups A9–A10 is illustrated The catecholamine type cells are indicated with dots *SNL* Substantia nigra pars lateralis For the other abbreviations see Text figs –4



Text fig 6 Transverse section just cranial to the nuc interpeduncularis The topography of groups A9–A10 is illustrated The catecholamine type cells are indicated with dots *CM* corpus mamillare *FRF* Fasciculus retroflexus For the other abbreviations see Text figs –5

Nerve cells of the 5 HT type

GROUP B7 This large group of closely packed small to medium sized cells was situated in the substantia grisea centralis The vast majority of the cells were localized within the nuc dorsalis raphe especially in the part just above and medial to the fasciculus longitudinalis medialis (Text fig 2 Fig 36) A number of cells however were present lateral to this nucleus and within the caudal portion of the nuc Edinger Westphal A few were also present lateral to the nuc n trochlearis and fasciculus longitudinalis medialis The cells were round to oval and their cytoplasm contained coarse intensely stained Nissl granules

GROUP B8 This group was fairly large and consisted of small to medium sized cells The cells were present from the caudal end of the posterior colliculus to the caudal third of the nuc interpeduncularis being situated mainly within the nuc medianus raphe (Text fig 2 Fig 37) Some were also present within the caudal portion of the nuc linearis In the cranial portion of the cell group a number were also found just lateral to the nuc medianus raphe within the formatio reticularis A few B8 cells were present also within the decussation of the pedunculus cerebellaris superior The cells had coarse to fine moderately stained Nissl granules

GROUP B9 This group was fairly large and was present mainly within and around the lemniscus medialis (Text fig 2 Fig 38) from the caudal end of the posterior colliculo to the caudal third of the nuc interpeduncularis A number were also present dorsal to the lemniscus medialis within the formatio reticularis mesencephalici The small to medium sized cells were round to oval and a number of them were clearly multipolar The Nissl picture was the same as for group B8 Especially the cells of group B7 were seen to send axons ventrally towards the nuc interpeduncularis These axons then made a sharp bend and ran parallel to the ventral surface of the brain dorsal to this nucleus mainly within the decussatio tegmenti ventralis and dorsalis At the junction between the mesencephalon and diencephalon some of them were concentrated in a tract just dorsomedial to the fasciculus retroflexus This tract then bent around the fasciculus retroflexus on its medial side and passed through the dorsolateral part of the mammillary body where several of its fibres probably terminated The fibres were lying mainly within the medial forebrain bundle and medial and parallel to the green fluorescent fibres also situated mainly within this bundle

Nerve cells of the catecholamine type

GROUP A11 A small group of scattered cells with a weak to strong green fluorescence was present close to the third ventricle medial dorsal and ventral to the fasciculus retroflexus mainly within the substantia grisea periventricularis but with a few cells also within the area of the nuc hypothalamicus posterior area supramammillaris and nuc reuniens thalami The cells were medium sized and round to oval

GROUP A12 This small group of small round nerve cells with a weak green fluorescence was situated within the nuc arcuatus especially its anterior portion and the ventral portion of the nuc periventricularis anterior (Fig 39) The cells had fine lightly stained Nissl granule This group which seems to send axons terminating in the region of the primary capillary plexus of the hypophysial portal system has been described earlier (FUXE 1964*a*)

The two different types of monoamine containing nerve cells have been found also in the other mammals studied No obvious differences have been seen but the material is small and insufficiently examined It is therefore impossible at the present time to exclude differences as regards the localization of the cells etc

III Histochemical criteria for the specificity of the fluorescence reaction

The fluorescence method is no empirical method with an obscure chemical basis It has been extensively studied for about three years with the use of model systems and with highly divergent tissues from both vertebrates and invertebrates (see e g FALCK HILLARP THIEME and TORP 1962 FALCK 1962 CARLSSON FALCK and HILLARP 1962 CARLSSON FALCK FUXE and HILLARP 1964 HAMBERGER and NORBERG 1963 MALMFORS 1963 CORRODI and HILLARP 1963 1964 FUXE 1964*a* DAHLSTROM and FUXE 1964*a* BERTLER FALCK and OWMAN 1963 DAHL FALCK LINDQVIST and MECKLENBURG 1962 FALCK HAGGENDAL and OWMAN 1963) The method has been found to have a very high sensitivity and specificity for catecholamines and 5 HT while their metabolites (the 3 O methylated amines and the phenolic acids) the tertiary amines and N acetyl 5 methoxy tryptamine (melatonin) do not give any significant fluorescence Closely related phenethylamines (tyrosine tyramine) are non reactive and indolethyl amines (tryptophan tryptamine) give weakly fluorescent products The amine precursors dopa and 5 HTP however develop about the same

fluorescence as the corresponding amines. The m-hydroxy derivatives (m-tyrosine, m-tyramine) and the corresponding O-methylated and/or β-hydroxylated compounds, on the other hand, have so far not been found to interfere with the localization of the normally occurring tissue monoamines, even if very large doses of the amino acids are administered. This in spite of the fact that they can under certain conditions develop fluorescence in models (HILLARP, personal communication). Some proteins—especially if the formaldehyde treatment is prolonged—may give a weak green to yellow fluorescence, which probably explains the somewhat disturbing background fluorescence sometimes observed in the tissue sections.

Under the conditions used in the histochemical method primary catecholamines such as DA, NA and 5-HT readily condense with formaldehyde and are converted to intensely fluorescent 6,7-dihydroxy-3,4-dihydroisoquinolines and 6-hydroxy-3,4-dihydro-β-carbolines, respectively (see CORRODI and HILLARP 1963, 1964). Secondary amines such as A require a higher temperature or longer times (e.g. 3 h at $+80$ C) and are thus easily differentiated from the primary ones (cf. FALCK, HÄGGENDAL and OWMAN 1963). Unfortunately the main peak of activation of the fluorescent compounds lies in the same wavelength range (390–410 mμ). The fluorescence spectrum of the β-carboline, however, shows a peak (510–520 mμ) which lies 30–40 mμ higher than that of the isoquinolines. This explains why the colour of the emitted light in a fluorescence microscope with a secondary filter with high absorption below 490 mμ appears respectively yellow and green to yellow-green.

It has further been found in several of the above-mentioned studies that the monoamines and their fluorescent compounds behave in essentially the same way in the tissue stores as in models. The conditions for the development of fluorescence in the nerve cells studied in the present paper and the properties of the fluorescent products contained in them should thus resemble those in models and cell structures known to contain respectively primary catecholamines and 5-HT if the fluorescence is due to the presence of these amines. This was studied in several ways with the use of sections from brain stems of untreated and nialamide-treated rats. All the histochemical criteria examined (see below) were found to be satisfied. The nerve cells with weak fluorescence could not, however, be tested in all respects.

1. Conditions for the development of fluorescence. When the reaction was performed for 1 h at 80 C no fluorescence was obtained if the paraformaldehyde was very dry. If the amount of adsorbed water was increased more and more (using the technique of HAMBERGER and MALMFORS 1964)

fluorescence first appeared with increased intensity then became indistinct and finally disappeared due to an increased diffusion A reaction time of about 1 h was sufficient to obtain maximal fluorescence when the formaldehyde gas had a water content somewhat less than that found to cause the beginning of diffusion If the fluorescence is specific the latter finding is strong evidence that it is developed from primary amines — Autofluorescent structures present in certain central and peripheral nerve cells and in many peripheral tissues showed no obvious changes when the reaction conditions were altered Any proteins that may become fluorescent will for obvious reasons not readily diffuse They will also probably quickly become more or less insoluble due to the high tanning action of wet formaldehyde gas

2 *Peak of activation and colour of the fluorescence* With the equipment used the fluorescent compounds in the nerve cells of the catecholamine type—like the compounds developed from catecholamines in other cells and models—showed a main peak of activation at 395–405 mμ and emitted a green to yellow green light Due to the rapid photo decomposition of the fluorescent product from 5 HT (see below) it was impossible to examine the nerve cells of the 5 HT type With the use of a new microspectrophotometric technique developed by CASPERSSON for the determination of spectra of fluorescent cell structures in tissue sections it has further been found that the fluorescence of the two types of nerve cells showed emission spectra which were practically identical with those of the fluorescent compounds formed in models and tissue sections from DA and NA and 5 HT respectively (CASPERSSON HILLARP and RITZEN 1964)

3 *Sensitivity to irradition with ultraviolet light* When the nerve cells were irradiated with UV light (HB 200 lamp 3 mm BG 12 filter) in the fluorescence microscope the yellow fluorescence decreased rapidly and markedly within one minute and was very strongly reduced after 10 to 15 min The green fluorescent compounds were much more resistant and showed a noticeable to marked decrease first after 45 to 90 min This wide difference in UV lability has been found also between the yellow and green fluorescence of the 5 HT and NA containing terminals in the spinal cord Using CASPERSSON s microspectrophotometric technique it has further been demonstrated that the fluorescent compounds developed from 5 HT and catecholamines show this difference both in models and in tissue sections (CASPERSSON HILLAPP and PITZÉN 1964) This simple test seems thus to be of great value for the differentiation between catecholamines and 5 HT —The very marked UV lability of the 5 HT product is undoubtedly the

main reason why microphotographs often do not show the strong fluorescence of the 5 HT cells in animals treated with a potent MAO inhibitor

4 *Quenching of the fluorescence by water* The fluorescent compounds from catecholamines and 5 HT show no or only a weak fluorescence in water or aqueous solutions of ethanol or isopropanol When enclosed in or bound to proteins in models or tissue sections (cf CORRODI HILLARP and JONSSON 1964) they show the same phenomena The fluorescence developed by formaldehyde treatment in the nerve cells—but not the autofluorescence—likewise strongly decreased or disappeared when the sections were mounted in 40 % isopropanol and reappeared after dehydration and mounting in xylene

5 *Reduction to non fluorescent compounds* It has recently been shown that the fluorescent compounds from catecholamines and 5 HT are very readily reduced by sodium borohydride under mild conditions to the corresponding 1 2 3 4 tetrahydro compounds These are non fluorescent but can be converted again to the fluorescent 3 4 dihydro-products on renewed formaldehyde treatment (CORRODI HILLARP and JONSSON 1964) Treatment with borohydride has so far never been found to cause obvious changes in the non specific fluorescence (e g autofluorescence protein fluorescence) found in peripheral tissues and nerve cells This histochemical test which is of high specificity has in the present paper been used to examine the fluorescence of the nerve cells in the brain stem It was found that 0 03 to 0 1 % sodium borohydride in 90 % isopropanol within 2 min at +20°C caused the green and yellow fluorescence—although not the autofluorescence or background fluorescence—to disappear and also that the fluorescence could be regenerated again by formaldehyde treatment This result strongly supports the view that the nerve cell fluorescence is specific

IV Pharmacological criteria for the specificity of the fluorescence reaction

It has been shown in previous studies with the fluorescence method that certain drugs and dopa analogs which either deplete the monoamine stores or increase the monoamine content in the central nervous system are of great value in evaluating the specificity of the method and also in differentiating between the various monoamines (see e g CARLSSON FALCK and HILLARP 1962 CARLSSON FALCK FUXE and HILLARP 1964 FUXE 1964*a*) Experiments similar to those used in these studies were therefore performed Since their significance has been discussed at length in previous papers (see also CARLSSON 1964) and a detailed account of all the pharmacological

experiments will be forthcoming in the next section and in following papers in this series there will be given below only a brief summary and commentary on certain data of importance The drugs used caused characteristic and marked changes in the fluorescence of the nerve cells which according to the histochemical findings contained monoamines No other obvious changes (e g in the autofluorescent structures) were observed

The fluorescence of the nerve cells was strongly reduced to completely abolished in animals treated with a large dose of reserpine which depletes all stores of their monoamines in the nervous system Disappearance was complete as early as 2 h after administration of the drug The nerve cells were thus affected about as rapidly as the monoamine containing terminals but the cell recovered (see Table 2) much more rapidly (cf CARLSSON FALCK FUXE and HILLARP 1964 FUXE 1964*b*)

The results (Table 3) obtained in the experiments with the two chemically different MAO inhibitors MO 911 (N benzyl N methyl propynyl amine) and nialamide (1 isonicotinyl benzylcarbamyl ethylhydrazine) and with reserpine nialamide give strong support to the view that the yellow fluorescence of the nerve cells in the B groups is due to the presence of 5 HT The same results were obtained with mice It is specially noteworthy that nialamide (500 mg/kg) given to animals previously treated with a large dose of reserpine (rat 10 mg/kg mouse 25 mg/kg) rapidly (5–6 h) caused the appearance of a strong yellow fluorescence not only in the cell bodies of these nerve cells but also over the entire length of the axons and their synaptic terminals (see also FUXE 1964*b*) In contrast to this the fluorescence of the Group A cells did not change and the many synaptic terminals which in normal animals show an intense green fluorescence but no fluorescence in reserpinized animals remained non fluorescent The significance of this is clear from the circumstance that nialamide given to mice previously treated with a large dose of reserpine affects the very low levels of the catecholamines and 5 HT in the brain and spinal cord quite differently DA and NA remain practically unchanged but the 5 HT is tremendously increased (CARLSSON LINDQVIST and MAGNUSSON 1960 CARLSSON personal communication)

The administration of *m* tyrosine caused a more or less marked decrease in the fluorescence of the Group A cells but no changes or a small increase in that of the B cells (Table 3) This analog of the catecholamine precursor tyrosine also brought about a disappearance of the synaptic terminals with green fluorescence but left the yellow fluorescent terminals unaffected Administration of the other analog α methyl *m* tyrosine *initially* caused a very large decrease in the fluorescence of the A cells (Text fig 8) but a

Changes in fluorescence intensity after reserpine treatment

Time after Reserpine	A1	Medulla oblongata A2	A4	Pons A5–A7	Mesencephalon A8–A10	Diencephalon A11	Diencephalon A12	Lower brain stem B1–B9
10 mg/kg ½ h	Moderately reduced	As group A1	Strongly reduced		As group A1–A7	As group A1–A7	As group A1–A7	No certain changes
1 h	Very strongly reduced	As group A1	Very strongly reduced (or abolished)		As group A1–A7	As group A1–A7	As group A1–A7	No certain fluorescence
2 h 4 h	Very strongly reduced to abolished	As group A1	Abolished (or very strongly reduced)		As group A1–A7	As group A1–A7	As group A1–A7	No fluorescence
6 h	Marked regeneration	Started regeneration	Started regeneration		As group A1–A7	As group A1–A7	As group A1–A7	No fluorescence
8 h	Marked regeneration up to normal or even increased levels in the cell body	Marked regeneration up to normal levels	Started regeneration		Started regeneration	Marked regeneration	Strongly reduced	No certain fluorescence
12 h	Clearly increased above normal in the cell body	Normal or slightly increased above normal	Regeneration not yet completed		Marked regeneration	Normal	Regeneration not yet completed	No certain fluorescence or normal
24 h	Markedly increased above normal		Regeneration almost completed		Regeneration almost completed	Normal	Regeneration almost completed	Normal
[illegible]	[illegible]							

48 h	Markedly increased above normal	Normal	Normal	Normal	Normal	Normal	Normal
7 -96 h	Normal or slightly increased (72 h)	Normal	Normal	Normal	Normal	Normal	Normal
5 mg/kg 4 h	Slightly increased	Normal	Regeneration almost completed	Regeneration almost completed	Normal	Regeneration almost completed	Normal
1 mg/kg 24 h, 0 1-0 2 mg/kg 24 h	Normal	Normal	Normal	Normal	Normal	Normal	Normal
5 mg/kg 4 h	Strongly reduced to abolished	As group A1	Abolished (or very strongly reduced)	As group A4-A7	As group A4-A7	As group A4-A7	No fluorescence
1 mg/kg 4 h	No certain changes or slightly reduced	As group A1	Weakly to moderately reduced	As group A4-A7	As group A4 A7	As group A4-A7	No certain fluorescence
0 1-0 2 mg/kg 4 h, 0 05 mg/kg 4 h	Normal	Normal	Normal	Normal	Normal	Normal	Normal

Table 2 Changes in fluorescence intensity after reserpine treatment

Time after Reserpine	Medulla oblongata		Pons	Mesencephalon	Diencephalon		Lower brain stem
	A1	A2	A4 A5 A7	A8 A10	A11	A12	B1 B9
10 mg/kg 1 h	Moderately reduced	As group A1	Strongly reduced	As group A4 A7	As group A4 A7	As group A4 A7	No certain changes
1 h	Very strongly reduced	As group A1	Very strongly reduced (in cell body)	As group A4 A7	As group A4 A7	As group A4 A7	No certain fluorescence
2 h} 4 h}	Very strongly reduced to abolished	As group A1	Abolished (or very strongly reduced)	As group A4 A7	As group A4 A7	As group A4 A7	No fluorescence
6 h	Marked regeneration	Started regeneration	Started regeneration	As group A4 A7	As group A4 A7	As group A4–A7	No fluorescence
9 h	Marked regeneration up to normal or even increase in the cell body	Marked regeneration up to normal levels	Started regeneration	Started regeneration	Marked regeneration	Strongly reduced	No certain fluorescence
12 h	Clearly increased above normal in the cell body	Normal or slightly increased	Regeneration not yet completed	Marked regeneration	Normal	Regeneration not yet completed	No certain fluorescence or normal
24 h	Markedly increased above normal	above normal	Regeneration almost completed	Regeneration almost completed	Normal	Regeneration almost completed	Normal

small increase in the B cells The reactions of the terminals were the same as those found on *m* tyrosine treatment These results support the view that the two types of fluorescence are due to the presence of catecholamines and 5 HT respectively — The reason why α methyl *m* tyrosine only caused a small decrease in Group A11 is under study It may be that these cells have an amine metabolism of such rapidity that they had almost recovered from an initial depletion On the other hand—since they showed only a small decrease on *m* tyrosine treatment—it may be that their uptake and/or decarboxylation of the analogs is too slow so that the decarboxylated products from the analogs do not accumulate in sufficient concentrations in the cells to cause a depletion of the endogenous amines (cf CARLSSON 1964)

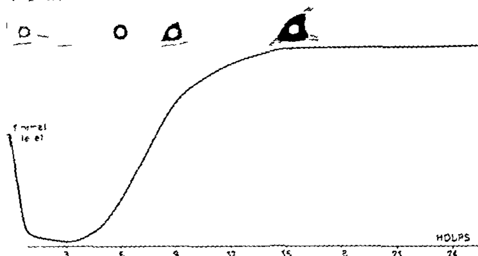
It may be concluded that the findings from the histochemical and pharmacological experiments performed in this study furnish when taken together convincing evidence that the fluorescence of the two types of nerve cells is due to the presence of catecholamines and 5 HT respectively

V Effects of drugs interfering with the monoamine metabolism

Since certain drugs—especially reserpine MAO inhibitors and dopa analogs—have proved very valuable in the elucidation of monoamine metabolism in the central nervous system it is of great interest to examine their effects on the monoamine containing neurons The drug induced changes in the fluorescence of the nerve cells present in the brain stem have therefore been studied

The yellow fluorescent nerve cells belonging to the different anatomical groups described above all reacted so similarly that the groups could not be clearly distinguished from each other The green fluorescent cells on the other hand were found to be a pharmacologically heterogeneous population the reactions of the cells were similar *within* the different anatomical groups but differed *between* the groups The cells of practically all the groups found in the brain stem have therefore been examined more closely The changes in the fluorescence of the cells are summarized in Tables 2 and 3 and Text fig 7 and 8 The drugs used their administration etc are given in Table 1 The findings are briefly commented below

Reserpine (Table 2 Text fig 7) The specific fluorescence of the nerve cells was markedly reduced as early as 30 min after the administration of a large dose of reserpine It was strongly reduced to abolished at 2 and 4 h when also the fluorescence of the monoamine containing nerve terminals had almost completely disappeared In contrast to this the auto



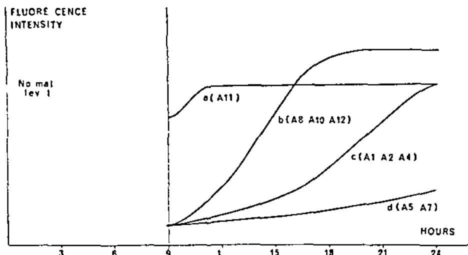
TEXT FIG. 7 Schematic illustration of the depletion and recovery of the catecholamines in the nerve cell bodies of group A1 following the administration of a large dose (10 mg/kg) of reserpine

The recovery starts rapidly with an amine accumulation in a zone around the nucleus. In the later stages amines begin to accumulate in the entire cell body and are finally found also in the processes. In the cells of group A1 the regenerative processes are intense and the amine content is increased considerably above the normal level after 12 to 15 h. This phenomenon is not observed in the catecholamine cells of the other groups which show a recovery up to normal amine levels after about 24 to 48 h. Otherwise they do not differ in principle from the A1 cells. ■ Strong fluorescence ▢ Medium fluorescence ▢ Weak fluorescence ▢ No fluorescence

fluorescent granules present in certain nerve cells have never been found to be affected by this drug

The different groups of green fluorescent cells showed, however marked differences with regard to the regeneration of fluorescence (Text fig. 7 and Table 2) Regeneration was quickest in the group A1 and A2 cells many of which showed normal or even increased fluorescence level (A1) as early as 8 hours after administration of the drug Group A11 showed a somewhat less rapid recovery while the other groups recovered their normal fluorescence picture somewhere between 24 and 48 hours The rank order of the groups in respect of the rapidity of their regeneration was as follows A1 A2 A11 A8–A10 A4–A7 and A12

As a general rule fluorescence first began to reappear in the zone around the non fluorescent cell nucleus (Fig 42) This was best seen in the cell of groups A1 A2 and A6 The intensity of the fluorescence in this zone



TEXT FIG 8 Schematic illustration of the time course of catecholamine recovery in different cell groups following treatment with α methyl m tyrosine in two doses of 400 mg/kg at an interval of _ h The earliest time studied after the first injection was 9 h (For further explanation see the text)

usually reached normal levels (or in group A1 even supernormal levels) before any more marked regeneration of the fluorescence was seen in the outer part of the cell body Last to regain their fluorescence were the cell processes (Text fig 7)

The cells of group A1 not only recovered their normal fluorescence rapidly but most of them (and some cells also in group A2) showed a distinct or even marked increase in intensity especially in the zone around the nuc leus 8 12 24 and 48 h after the administration of reserpine (Figs 42–44) In group A1 the fluorescence did not return to normal levels until after 72–96 hours Those cells which had a marked increase in their fluorescence also appeared to be larger and more swollen than usual There is little doubt that the increase in fluorescence intensity was due to an increased content of a primary catecholamine the fluorescence spectrum was the same as that of normal cells and the histochemical criteria were fulfilled When lower doses of reserpine were used the increase in the fluorescence was not observed

The sensitivity of the nerve cells to reserpine was al o tested The animals were killed 4 h after the administration of 0 05 0 2 1 and 5 mg/kg The largest dose was as effective as 10 mg/kg while the two lowest doses did not give any obvious changes in the nerve cell fluorescence In the animals

given 1 mg/kg the cells in pons and mesencephalon showed a medium fluorescence reduction which affected all parts of the cell bodies and their processes while the cells in medulla oblongata were normal or slightly decreased in fluorescence intensity

MAO inhibitors (Table 3) The fluorescence of the green fluorescent nerve cells showed no certain changes or a slight increase after a large dose of malamide iproniazide or MO 911 When very large doses (400 mg/kg) of MO 911 were used and especially when the animals were kept at +29°C for 24 to 44 h green fluorescent material had accumulated around the cell bodies which therefore appeared indistinct This may indicate that catecholamines had been released since there is other evidence that MAO inhibitors in high doses may give rise to an amine release in the brain (CARLSSON LINDQVIST and MAGNUSSON 1960) Another phenomenon observed in these animal seems to give direct evidence for a release The normally intense fluorescence of the catecholamine containing nerve terminals in the brain stem had become indistinct or actually disappeared and the areas in which the terminals are highly concentrated showed instead a diffuse weak fluorescence of the specific green type due in all probability to the presence of primary catecholamines in the tissue surrounding the terminals (The deaminated or O methylated metabolites of the catecholamines do not give any fluorescence reaction) The 5 HT cells and terminals showed similar signs of release—although to a lesser degree

The nerve cells that in untreated animals have a very weak to weak yellow fluorescence showed a very strong increase in intensity 4 to 6 hours after the administration of malamide or MO 911 (Figs 7–10) Many nerve cells that normally have no certain fluorescence appeared at the same time with a strong yellow fluorescence There is little doubt that this was due to an increased content of a monoamine of the 5 HT type malamide and other potent MAO inhibitors give a marked increase in the brain 5 HT the fluorescence spectrum was the same as that of the fluorescent product formed from 5 HT and the histochemical criteria were fulfilled Since these cells were found to belong to the groups that in untreated animals contain nerve cells with very weak to weak yellow fluorescence it seems evident that the 5 HT neurons normally have such low concentrations of the amine in their cell bodies that only a part of them can be detected with the histochemical method used

The axons and larger dendrites that belong to the 5 HT neurons, but which cannot normally be observed showed a yellow fluorescence of medium to strong intensity in the animals treated with malamide (Figs 8 11) It thus became possible to trace axons for long distances from the cell bodies

and in some cases all the way to their terminals (DAHLSTRÖM and FUXE 1964a and b)

The 5 HT neurons showed a progressive increase in fluorescence above all around the nucleus 1 to 2 h after the administration of MO 911 (300 mg/kg) After 3–4 h however the 5 HT neurons had a strong yellow fluorescence which means that a fairly large increase in 5 HT had occurred in the cells between 2 and 4 h after administration of the drug Now also there could be seen the larger dendrites and the axons which had not been observed 1–2 h after administration Also the yellow fluorescent terminals showed up much better Up to 2 h after administration the terminals had been of normal appearance The effect of iproniazide was qualitatively the same but much less marked than that of nialamide and MO 911

Reserpine–nialamide (Table 3) The green fluorescent cells showed about the same appearance in animals treated with reserpine–nialamide as in those treated with reserpine alone The fluorescence of the catecholamine containing nerve terminals did not reappear in reserpinized animal after the administration of nialamide

In contrast to this nialamide elicited the same very strong increase in the fluorescence of the 5 HT neurons both in reserpinized and non reserpinized rats (Figs 6–12 20–22 36–38) The fluorescence of the axons was in fact even more apparent in the reserpinized animals perhaps due to a higher contrast having been achieved with the disappearance of the intense green fluorescence in the catecholamine terminals Also the terminals belonging to 5 HT neurons regained a fairly strong yellow fluorescence when nialamide was given to reserpinized animals This will be described in detail in the next two papers in this series (DAHLSTRÖM and FUXE 1964b FUXE 1964b)

Nialamide dopa The background fluorescence was markedly increased and was clearly green 30 min after the administration of a large dose of L dopa to animals pretreated with nialamide It seems probable that this was due to the presence of dopa itself in the brain tissue (cf CARLSSON and HILLARP 1962) The endothelium of the brain capillaries showed a strong green fluorescence

No obvious changes in the green fluorescent cells present in the medulla oblongata and pons were observed after the injection of dopa but a small increase in fluorescence intensity would probably have been masked by the background fluorescence A medium increase in the green fluorescence was found in the nerve cells of groups A8–A10 The cells showed an indistinct outline due to the presence of a zone with green fluorescence around the cell bodies It may be that amines are formed from dopa so rapidly that

they leak out in detectable amounts This may also explain the finding that the catecholamine containing cells were indistinct and that the terminals were difficult to observe

The strong yellow fluorescence induced by nialamide in the 5 HT nerve cells showed no obvious changes after dopa injection The intensity was possibly somewhat lower than in animals treated with nialamide only These cells in contrast to the green fluorescent cells had a distinct outline and no accumulation of green fluorescent material could be observed around them

Nialamide 5 HTP The background showed a weak yellow fluorescence probably due to the presence of the amino acid in the tissue The endothelium of the brain capillaries displayed a distinct yellow fluorescence The catecholamine containing nerve terminals showed up better than in the animals treated with nialamide dopa

No obvious changes were observed in either the green or the yellow fluorescent cells No accumulation of fluorescent material around the cells could be detected

Meta tyrosine (Table 3) The background showed a weak green fluorescence probably due to the presence of *m* tyrosine in the tissue (cf CARLSSON FALCK and HILLARP 1962) The catecholamine containing nerve terminals showed no or only a weak fluorescence but the 5 HT terminals seemed unaffected

The fluorescence of the green fluorescent cells was decreased to a greater or lesser extent The rank order of the cell groups as regards the extent of this decrease was as follows A8 A9 and A12 > A10 > A1 A2 and A4–A7 > A11 The cells in A8 A9 and A12 were thus affected most their fluorescence could often hardly be detected and in some of them may well have been abolished

The 5 HT cells were unchanged or somewhat increased in fluorescence intensity around the nucleus

α Methyl m tyrosine (Text fig 8) No increase was observed in background fluorescence Following the administration of two large doses of α methyl *m* tyrosine the green fluorescent terminals had disappeared to a very large extent 9 h after the injection of the first dose Most of them had not recovered any fluorescence by 24 h In some areas however many terminals regained their normal intense fluorescence 18 to 24 h after the injection The former terminals in all probability contain NA the latter probably DA (cf CARLSSON FALCK and HILLARP 1962)

As seen in Text fig 8 all cells of the catecholamine type showed a large decrease 9 h after the first injection except for the cells of group A11

which showed only a small decrease at this time After this time there could be observed four different types of recovery curves (curves a b c and d) The cells of groups A5–A7 showed the slowest recovery and were still markedly reduced 24 h after the administration of even a single dose (curve d) The cells of groups A1 A2 and A4 on the other hand had by this time (24 h) recovered their normal intensity (curve c) The cells in mesencephalon (A8–A10) and in hypothalamus (A12) however had recovered their fluorescence much earlier and showed a normal or even somewhat increased fluorescence intensity 15 to 18 h after the first injection (curve b) (Figs 39 40) The cells of group A11 were of normal appearance as early as 12 h after the first injection (curve a)

The 5 HT cells showed no distinct changes after a single dose of the drug but the administration of two doses led—in those cases studied—to a small to medium increase in the yellow fluorescence especially around the nucleus The yellow fluorescent terminals also showed up better but this was probably due to the fact that most of the terminals with an intense green fluorescence had disappeared

Most of the biochemical studies on the effects of drugs on brain monoamines which are of special relevance for the experiments described above have been made on mice by Dr A CARLSSON s group

The effects of nialamide (500 mg/kg 5–6 h) in normal and reserpinized (25 mg/kg 18 h before the administration of nialamide) mice were therefore examined The results obtained did not differ—at least qualitatively—from those reported above

The findings from the histochemical and pharmacological experiments furnish convincing evidence that the two principally different types of nerve cells with specific fluorescence contain primary catecholamines and 5 HT respectively There is other evidence for this view

(a) Of the monoamines which—under the conditions used—can be converted to products with green to yellow fluorescence only DA NA and 5 HT have been found in significant amounts in the brain stem The content of A is too low (see BERTLER and ROSENGREN 1959 GUNNE 1962) to interfere

(b) There is no evidence that the amino acid precursors dopa and 5 HTP which give the fluorescence reaction exist in sufficiently high concentrations in monoamine forming cells to interfere (see HALL HILLARP and THIEME 1961 CARLSSON and HILLARP 1962) That these intermediate metabolites are not normally accumulated in the cells is indicated also by the observations that the first step in the amine synthesis is in all probability rate limiting (cf BERTLER HILLARP and ROSENGREN 1960 UDENFRIEND and ZALTZMAN-NIRENBERG 1963 AXELROD 1963 CARLSSON CORRODI and WALDECK 1963)

(c) Tetrabenazine which is chemically quite different from reserpine but—like this drug—depletes the brain stores of their monoamines (see PLETSCHER BROSSI and GEY 1962) has been found to cause a rapid disappearance of the specific fluorescence of the nerve cells (BARTONIČEK DAHLSTROM and FUXE unpublished data) MAO inhibitors (transleypromine Catron) that are chemically different from those used in the present study but which give a pronounced increase in the brain 5 HT levels were also found to produce a marked increase in the specific yellow fluorescence in both cell bodies and axons of the 5 HT neurons

It has in fact been possible to obtain an almost conclusive characterization of three of the neuron systems discovered

The finding that the substantia nigra (CARLSSON 1959 BERTLER 1961) and especially the zona compacta (HORNYKIEWICZ 1963) has a fairly high DA content and—as demonstrated by ROSEGAY (1944)—probably give rise to nerve fibres terminating in the very DA rich neo-striatum (BERTLER and

ROSENGREN 1959, CARLSSON 1959) strongly suggested that the large number of specific nerve cells (group A9) discovered in this area and found to contain a primary catecholamine are DA neurons. It has now been experimentally shown that DA in the neo-striatum is accumulated in very high concentrations in nerve terminals arising from the axons of these cells (ANDÉN, CARLSSON, DAHLSTRÖM, FUXE, HILLARP and LARSSON 1964). The fact that the nerve cells are numerous and—as compared with the peripheral adrenergic ganglion cells (HAMBERGER and NORBERG 1963)—seem to have a relatively high amine content probably explains why markedly higher concentrations of DA are found in the mesencephalon (DA = 0.24, NA = 0.52 $\mu g/g$) than in the pons plus the medulla oblongata of the rat (DA = 0.08, NA = 0.48 $\mu g/g$) (ANDÉN, unpublished data). Since most—if not all—of the A9 cells have been found (unpublished observations) to send their fibres to the neo-striatum and the rat hypothalamus contains not inconsiderable amounts of DA (DA and NA about 0.4 and 1.5 $\mu g/g$ respectively, data obtained by ANDÉN) ascending DA systems may arise from some of the other catecholamine cell groups in the lower brain stem. The nigro-neostriatal DA neurons, however, form the dominant system as seen from the fact that 80 per cent or more of the brain's entire DA content occurs in the caudate nucleus and putamen (CARLSSON 1961).

It has similarly been demonstrated that the NA and 5-HT found in the spinal cord are accumulated in very high concentrations in synaptic terminals (CARLSSON, FALCK, FUXE and HILLARP 1964) and that these terminals belong to axons descending in the lateral and anterior funiculi and originating—at least partly—from catecholamine (group A1) and 5-HT (group B1 and B2) containing nerve cells in the medulla oblongata (DAHLSTRÖM and FUXE 1964b).

These results taken together with those from the histochemical and pharmacological experiments furnish almost conclusive evidence that the neurons of these three systems are monoamine neurons with specific formation and storage of DA, NA and 5-HT respectively. Since the fundamental characteristics of the nerve cells belonging to the different catecholamine and 5-HT cell groups present in the brain stem are principally similar *there seems to be little doubt that all the new neurons specifically produce and store either DA, NA or 5-HT and give rise to synaptic terminals where the amines show a tremendous accumulation.*

The mammalian brain thus contains specific DA, NA and 5-HT neurons. Such neurons have also been found in the brain stem of birds (FUXE and LJUNGGREN 1964). The first direct evidence for the existence of DA-containing nerve cells in mammals (in the retina) was obtained by HAGGENDAL and

MALMFORS (1963) There is also some evidence that the specific nerve cells in the arcuate nucleus and the ventral portion of the anterior periventricular nucleus (group A12) of the hypothalamus which seem to give nerve terminals to the primary capillary plexus of the hypophysial portal system are of the DA type (FUXE 1964a)

Of great interest in this connection are the findings that peripheral sympathetic neurons in the frog contain A instead of NA and thus give rise to truly adrenergic nerves (FALCK HÄGGENDAL and OWMAN 1963) and that both DA and 5 HT are localized to neurons in some invertebrates (DAHL FALCK LINDQVIST and MECKLENBURG 1962) There is furthermore reason to believe that the two latter amines act as transmitters in molluscs

As is well known the classical studies of EULER and co workers have shown NA to be the transmitter of the peripheral adrenergic nerves in mammals Strong evidence was also obtained for the view that this amine is present in the entire adrenergic neuron and stored in very high concentrations in the terminal part of the axons (see EULER 1956 1961) The introduction of the fluorescence method of FALCK and HILLARP made it possible to demonstrate directly the localization and intraneuronal distribution of the transmitter (FALCK 1962 1964 HAMBERGER and NORBERG 1963 HAMBERGER NORBERG and SJOQVIST 1963 1964 NORBERG 1964) Extensive studies have further been made in this laboratory on the uptake formation and storage of the transmitter its inactivation by MAO the intraneuronal localization of MAO the formation and transport of the amine storage granules the effects of axotomy and drugs etc (HAMBERGER MALMFORS NORBERG and SACHS 1964 DAHLSTROM and FUXE 1964d DAHLSTROM 1964 DAHLSTROM FUXE and HILLARP 1964 HILLARP and MALMFORS 1964 and unpublished data) It has thus been possible to examine certain fundamental properties and characteristics of the adrenergic neuron at the cellular level The monoamine neurons in the central nervous system have been studied in similar ways and have so far been found to have the same basic characteristics Since much of this work has not yet been published and the results obtained will be discussed at length in later papers in this series there will be made below only certain comments

The cell bodies of the peripheral adrenergic neurons have been found to contain variable low to very low amounts of NA (probably about 20 to 100 $\mu g/g$) evenly distributed throughout the cytoplasm The postganglionic axons also have low concentrations but dramatic changes occur when they are transformed into terminals within the innervated tissues the axons become thin abundant rounded or elongated enlargements (varicosities) appear along the whole length of the terminals and the concentration

of the transmitter is increased tremendously. Especially the varicosities contain the NA stored in the terminals and it seems probable that the transmitter is released—mainly at least—from these specialized structures. There is good evidence that NA is stored chiefly in the amine granules extensively studied by EULER and co-workers (see STJÄRNE 1964) and that these granules are formed in the cell bodies and then transported down to the terminals. As is well known, the specific storage mechanism is blocked by reserpine (cf. CARLSSON 1964) and the neuron contains the enzyme systems necessary for the formation of NA.

The monoamine neurons in the central nervous system have the same intraneuronal distribution of the amines. It is especially significant that the amines are stored in very high concentrations in the varicosities of the synaptic terminals that have the same appearance as the peripheral adrenergic terminals (cf. CARLSSON, FALCK, FUXE and HILLARP 1964). The cell bodies, on the other hand, have low to very low amine amounts comparable to those in the adrenergic ganglion cells. There is evidence that the amines are bound to storage granules which are formed and transported as in the peripheral neurons (see below). Both the peripheral and central neurons react in the same typical way to axotomy (DAHLSTRÖM and FUXE 1964*b*, *c*, *d*). The whole neuron, including its synaptic terminals, has not only a specific storage mechanism that is blocked by reserpine but also the capacity to synthesize the amine in question (see below).

There is thus no doubt that DA, NA and 5-HT in the central nervous system exist in neurons that have basic properties and characteristics similar or identical to those of the peripheral sympathetic adrenergic neuron in all respects examined. This strongly supports the view that the specific central neurons are monoaminergic, i.e. function by release of their amines from the synaptic terminals. Consequently, DA, NA and 5-HT may act as transmitters. Further evidence for this view has, in fact, been obtained recently from studies of the effects of dopa and 5-HTP on certain spinal inhibitory mechanisms (ANDÉN, LUNDBERG, ROSENGREN and VYKLICKY 1963; ANDÉN, CARLSSON and HILLARP 1964). Of special significance, however, is the finding that electrical stimulation of the descending systems of the spinal cord *in vitro* resulted in release and increased synthesis of 5-HT (ANDÉN, CARLSSON, HILLARP and MAGNUSSON 1964).

From a pharmacological point of view the 5-HT neurons differ in some respects from the catecholamine neurons. They reacted as a much more homogeneous population and all the MAO inhibitors tested caused a much more marked increase of the amine levels in the whole neuron. Most striking was the finding that 5-HT—in contrast to DA and NA—rapidly accumul

ated in the cell bodies axons, and terminals alike when a potent MAO inhibitor was administered to animals previously depleted of their mono amines by a large dose of reserpine No satisfactory explanation has been found for this phenomenon (CARLSSON LINDQVIST and MAGNUSSON 1960 CARLSSON 1964) but it does not necessarily imply a fundamental difference between the 5 HT and catecholamine neurons It may reflect differ ences in synthesis rates MAO activities or availability of MAO to the inhi bitors or indicate that MAO is of higher importance for the 5 HT metabo lism The 5 HT neurons in the brain of the pigeon normally show higher amine levels in their cell bodies than mouse and rat neurons and no such pronounced increase in 5-HT was found on MAO inhibition (FUXE and LJUNGGREN 1964) This problem will be further discussed in the third paper of this series

In contrast to the 5 HT neurons the catecholamine nerve cells proved to be a more heterogeneous population Marked differences were found between the various cell groups especially as regards the depletion and re covery of the amines in the cell bodies following the administration of *m* tyro ine α methyl m tyrosine and reserpine Of special interest is that the DA neurons (group A9) and the catecholamine neurons of the two other large groups (A8 and 10) in the mesencephalon (and also the A12 cells which may be DA neurons) were the most strongly affected by *m* tyrosine recovered most rapidly after α methyl m tyrosine and showed signs of amine release after the admini tration of dopa to nialamide treated animals Since the dopa analogs must be taken up and decarboxylated in the nerve cells in order to bring about their action (see CARLSSON 1964) these findings suggest that such neurons have a high amine metabolism The fact that recovery after a reserpine depletion was slow in the cell bodies of these cells does not contradict this view since this recovery in all probability reflects the formation of new storage granules (see below)

The marked differences found between the various cell groups—and especially between the cell bodies and the synaptic terminals—as regards rates of depletion or recovery and sensitivity to drugs must be emphasized also because of the distinct possibility that these differences give rise to serious sources of error in experiments based on determinations of the mono amine content or turnover in the entire brain or parts of the brain stem with a view to elucidate e g the relationships between changes in amine levels and other effects induced by drugs or the rate of amine synthesis Significant amounts of catecholamines will thus be found for instance in extracts of the lower brain stem especially the mesencephalon 24–48 h after a depletion by reserpine but this obviously has little to do with functional

recovery since the amines determined will originate from cell bodies and not from synaptic terminals

The rapid and large increase in the amine levels of the whole 5 HT neuron following the administration of potent MAO inhibitors to normal or reserpinized mice or rats and the finding that this increase is obtained also after axotomy (up to some days after the lesion unpublished data) indicate that the enzyme systems necessary for the 5 HT formation are present and have high levels of activity in the whole neuron They also strongly support the view (see CARLSSON and HILLARP 1962 CARLSSON 1964) that MAO is localized within the amine producing brain cells There is also good evidence that MAO is present in the peripheral adrenergic neuron (AXELROD 1963 KOPIN and AXELROD 1963 HAMBERGER MALMFORS NORBERG and SACHS 1964 and unpublished histochemical observations made by NORBERG)

Of considerable interest are the effects of reserpine which acts specifically by blocking the monoamine storage mechanism The cell bodies required higher doses than the synaptic terminals for a depletion but recovered much more rapidly The peripheral adrenergic neuron reacts in the same way (NORBERG unpublished data) The fact however that both cell bodies and terminals were depleted about as rapidly—and very strongly—indicates that reserpine blocks the storage mechanism also in the cell bodies This is further supported by the finding that tetrabenazine causes a similar depletion The specific action of this drug is also a blockade of the storage function but tetrabenazine—in contrast to reserpine—is a reversible short lasting blocker (see PLETSCHER BROSSI and GEY 1962) As discussed in other papers (DAHLSTRÖM FUXE and HILLARP 1964 HILLARP and MALMFORS 1964) there is strong evidence for the view that reserpine acts specifically and primarily on the Mg^{++} ATP dependent uptake–storage mechanism in the amine granules and not on the cell membrane (CARLSSON HILLARP and WALDECK 1963) This view readily explains—and is in fact strongly supported by—the findings that the cell bodies require higher doses of reserpine than the terminals that they recover much more rapidly and that the new formed amines accumulate during the recovery phase in the peculiar way shown schematically in Text fig 7 It was found that the catecholamines also in the nerve cell bodies of normal animals often show an uneven distribution with a higher content in a zone around the nucleus Amines were observed after a depletion induced by reserpine to be rapidly produced in this zone where they could accumulate to high concentrations before any amines were detected in the peripheral part of the cell bodies and very long before any recovery of the storage function in the nerve terminals occurred even after much lower doses of reserpine These and the other observations

discussed above—when considered together with the wealth of information on the formation storage and inactivation of the brain monoamines obtained from the extensive biochemical and pharmacological research carried out during the past 8 years—seem to show that the monoamine neurons synthesize their amines and store them in amine granules with a specific reserpine sensitive storage mechanism which explains their protection from MAO and their distribution within the cell bodies They further seem to indicate that the amine granules are formed in the zone around the nucleus and then presumably transported down to the terminals and that the granule formation is a fairly rapid process This process however shows markedly different rates between the various cell groups and may be stimulated by the administration of reserpine This explains both the peculiar intracellular distribution of the amines formed during the recovery phase after reserpine and why the cell bodies—in contrast to the terminals—show a rapid recovery in spite of the fact that reserpine produces a long lasting block of the storage function Experiments with transections or constrictions of the axons belonging to monoamine neurons have furnished independent evidence for the view that amine granules are formed in the cell bodies and transported down in the axons (DAHLSTROM and FUXE 1964*b–d*)

Administration of α methyl m tyrosine produces a long lasting depletion of NA—but not of DA or 5 HT—in the brain and this dopa analog has therefore been used to differentiate between DA and NA in histochemical studies on their cellular localization (see e g CARLSSON FALCK and HILLARP 1962) It was observed in the present study however that the cell bodies of both the DA neurons and the other catecholamine neurons in the mesencephalon recovered very rapidly and that ˜A nerve cells in the medulla oblongata (group A1) had about norm levels a h as after 24 h This may be due to a high amine n in the c ies of the mesencephalic neurons (see above) and rmati n amine granules in the A1 cells ' wei reco reserpine It would thus seem ı ts tool for differentiation between neurons

Several other findings conce to drugs are of interest The s nerve terminals and cell bodie after the administration of a po since there is biochemical evid hibitors is due to released ami

a distinct possibility that monoamines released from synaptic terminals in the central nervous system can be directly visualized. The pharmacological studies will be extended and discussed in detail in the third paper of this series however which will deal with the synaptic terminals belonging to the monoamine neurons. The numerous data which have accumulated during the past 8 years from biochemical, cytochemical, pharmacological and other investigations on the occurrence, subcellular localization, metabolism and function of the brain monoamines will also, insofar as they are relevant to our work, be discussed in that paper.

The cell bodies of the monoamine neurons seem to be localized almost exclusively in the lower brain stem. Only a few small groups of catecholamine cells have been found in the hypothalamus and posterior thalamus. *The NA and 5-HT fibres (no DA systems exist) in the spinal cord thus all descend from the medulla oblongata (and possibly higher levels) and it seems probable that most of the numerous systems of monoamine terminals present in the different parts of the brain derive from axons belonging to the special neuron groups in the lower brain stem.*

Most of the catecholamine cells and perhaps the larger part of the 5-HT cells are localized to 3 large groups each in the mesencephalon. Many of these cells—apart from the DA cells belonging to the nigro-neostriatal system—undoubtedly give rise to ascending fibre tracts. Fibre bundles belonging to both catecholamine and 5-HT neurons have in fact been found to ascend, for example, in the medial forebrain bundle (see also below). This no doubt explains the fall in brain 5-HT following lesions of this bundle in the rat (HELLER, HARVEY and MOORE 1962; HARVEY, HELLER and MOORE 1963).

It is of great interest that the cell groups of the "limbic midbrain area" (see NAUTA 1963)—except the ventral and dorsal reticular nuclei of Gudden —contain numerous catecholamine and 5-HT cells. This area gives rise to tracts which constitute an ascending link in a phylogenetically old integration system and seem to terminate mainly in the amygdaloid cortex and gyrus hippocampi, most of them undoubtedly via intermediate relay regions at various points along the medial forebrain bundle (cf. NAUTA 1963). Since numerous monoamine containing terminals have been found in these two structures and other parts of the limbic system (the septal area and the preoptic region) (unpublished data), there exists suggestive evidence that monoaminergic neurons with their cell bodies especially in groups A10, B7 and B8 play an important role as a part of this old integration system. Furthermore, there exist abundant ascending afferents from the reticular formation which via the medial forebrain bundle run to e.g. the amygdaloid

nuclei the septal area and the preoptic region The monoamine fibres in these parts may originate from the specific nerve cells present al o in the reticular formation This supports the view that monoamine systems ascending from the lower brain stem are an important part of the afferent link to the limbic system and are phylogenetically old

The localization of the 5 HT nerve cells is entirely different from that of the catecholamine cells They are found almost entirely in the raphe nuclei The significance of this is obscure since the connections and—especially—the functions of these nuclei are more or less unknown (TABER BRODAL and WALBERG 1960 BRODAL TABER and WALBERG 1960 BRODAL WALBERG and TABER 1960) The presence of numerous 5 HT cells in all parts of the raphe complex except in the two most rostral nuclei is of interest however, in that this complex—according to BRODAL *et al*—seems to be a primitive part of the brain stem which shows relatively little differentiation during the phylogenetic ascent of the vertebrates and may thus have relatively simple but fundamental and important tasks in the function of the brain (TABER BRODAL and WALBERG 1960) Some of the nuclei (nuc raphe obscurus pallidus magnus and pontis) give descending fibres to the spinal cord At least the first two of them and probably also the nuc raphe magnus contain 5 HT cells belonging to the descending 5 HT systems in the spinal cord BRODAL *et al* have shown that ascending fibres arise from all the raphe nuclei and that they seem to be links in ascending pathways from the spinal cord to higher levels 5 HT neurons may thus be a part of such links Of particular interest is the presence of 5 HT cells (group B7–8) in the rostral raphe region Fibres from this region seem to run to e g the neo striatum which has a relatively high content of 5 HT This 5 HT may consequently be localized in synaptic terminals deriving from 5-HT cells in the mesencephalon

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SUMMARY

The cellular localization of the monoamines in the brain stem of the rat—and to some extent also other mammals—has been studied with the use of a highly sensitive and specific fluorescence method and a pharmacological approach. The histochemical procedure and improvement for the standardization of the technique and elimination of important sources of error are described in detail. Rigorous histochemical and pharmacological criteria were used to ascertain the specificity of the fluorescence reaction and for the differentiation between the amines. Some new important specificity tests developed in this laboratory have been applied and a full account of the histochemical examinations is given.

Monoamines were found to be present in specific neurons of two distinctly different types, which in their cell bodies contain low amounts of either a primary catecholamine or 5-HT. The nerve cells are localized in many smaller or larger cell groups, which give rise to different ascending or descending fibre tracts. Experiments together with Dr. A. Carlsson's research group and Dr. K. Larsson (Depts. of Pharmacology and Psychology, University of Göteborg) have furnished data making it possible to obtain an almost conclusive characterization of three of the discovered neuron systems: one large nigro-neostriatal and two bulbo-spinal systems which produce and store DA, NA and 5-HT respectively. The amines show low concentrations in the cell bodies and processes. When the axons are transformed into synaptic terminals, however, dramatic changes occur: they become thin, abundant rounded or elongated enlargements (varicosities) appear along their whole length, and the concentration of the amines is increased tremendously. Especially the varicosities which may establish the synaptic contacts (see CARLSSON, FALCK, FUXE and HILLARP 1964) store large amounts of the amines.

Since the fundamental characteristics of the nerve cells belonging to the various catecholamine and 5-HT cell groups in the brain stem are principally similar, there seems to be little doubt that all the new neurons specifically produce and store either DA, NA or 5-HT and give rise to synaptic terminals where the amines show a tremendous accumulation.

The cell bodies of the monoamine neurons seem to be localized almost exclusively in the lower brain stem especially the mesencephalon Only a few small groups of catecholamine cells have been found in the hypothalamus and posterior thalamus Thus the NA and 5 HT fibres (no DA system exists) in the spinal cord all descend from from the medulla oblongata (and possibly higher levels) and it seems probable that most of the numerous systems of monoamine terminals in the different parts of the brain derive from axons belonging to the special nerve cell groups in the lower brain stem

The localization of the 5 HT nerve cells is entirely different from that of the catecholamine cells They are present almost exclusively in the various raphe nuclei while the latter cells occupy a more lateral position except in the cranial part of the mesencephalon The limbic midbrain area contains numerous cells of both types and many catecholamine cells exist in the mesencephalic reticular formation The possibility is discussed that monoamine neurons belong to phylogenetically old brain stem systems with primitive but fundamental functions They seem to be *inter alia* a part of the afferent link to the limbic system where monoamine terminals are abundant

The monoamine neurons in the brain stem have been examined and compared with the peripheral sympathetic adrenergic neuron as regards general morphology intraneuronal distribution formation and storage of the amines reactions to drugs interfering with amine metabolism and to axotomy The investigations have given information on *inter alia* the formation of storage granules in a zone around the cell nucleus the cellular localization of MAO and the site of action of reserpine Since the results will be discussed in detail in the forthcoming papers of this series however only the *general conclusion* is included in this summary

Three monoamines—DA NA and 5 HT—exist in the central nervous system in specific neurons which have similar or the same basic properties and characteristics in all respects examined as the peripheral adrenergic neuron This strongly supports the view that the specific central neurons are monoaminergic i e function by release of the amines from the synaptic terminals Consequently DA NA and 5 HT may act as transmitters in the central nervous system — There is other evidence for this view Of special significance is the recent finding that electrical stimulation of the descending systems in the spinal cord *in vitro* resulted in release and increased synthesis of 5 HT (ANDEN CARLSSON HILLARP and MAGNUSSON 1964)

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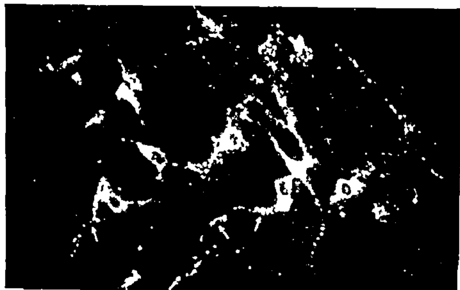


Fig 1 Group A1 Normal rat Transverse section Medium sized multipolar nerve cells with a diffuse green fluorescence of medium intensity are present in the caudal portion of the group Fine varicose green fluorescent fibres can be seen to make close contact with the fluorescent processes (↗) of the cells 2[illegible]0



Fig 2 Group A1 Normal rat Transverse section A medium sized spindle shaped nerve cell with two long green fluorescent processes and a diffuse green fluorescence in the cell body of strong intensity 165

Fig. 3. Group A. Normal rat. Transverse section. A number of small to medium sized round to oval cells of medium green fluorescence (A) can be seen mainly within the area of the nucleus commissuralis (A). The abundant supply of fine varicose green fluorescent fibres present in this area does not appear in the picture. Nucleus gracilis (*B*) is present ventrally, the central canal (*C*) medially, and the tractus solitarius (*D*) laterally. [illegible]



Fig. 4. Group A. Normal rat[illegible]. Transverse section. A [illegible] fine but [illegible] green fluorescence [illegible] with the [illegible] within the [illegible] only a few (*B*) of green fluorescent fibres present in this region actually app[illegible]

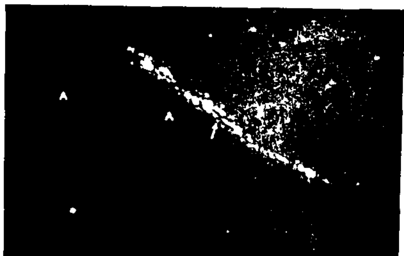


Fig 5 Group A4 Normal rat Tran verse secti n A row of small to medi im siz l r un l to oval nerve cells of medium green flu resc nc t ituated in the roof of the fourth ventricl ju t un l r the epen lyma ( ) Tl e pl xu cl r i l u is present at 1 × 100



Fig 6 Gro ip B1 Reserpine–nialami le treated rat Tran erse sect on A number of mall t i h n s zed o al v ll w fluore cent ner e c ll of m l um inten it are s tuate i tl medial l of tl tractus pyrami lal (A) The v ntral urface of tl l rain n licat l at B 100

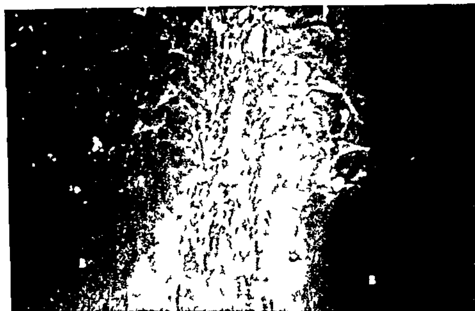


Fig. 7. Group B1. Nialamide treated rat. Transverse section. A number of closely packed oval and sometimes multipolar small to medium sized cells with a yellow fluorescence of medium to strong intensity are situated mainly within the cranial part of the nuc. raphe pallidus (A) partly between the pyramidal tracts (B). The cells are in close contact with fine varicose green fluorescent fibres (A). × 250

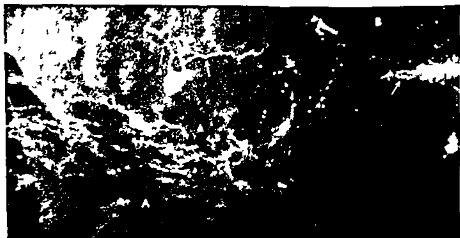


Fig. 8. Group B1. Nialamide treated rat. Transverse section. A few medium sized multipolar cells of medium to strong yellow fluorescence are present under the ventral surface of the medulla oblongata (A) and lateral to the pyramidal tract. The cells have fine fluorescent processes. Green fluorescent varicose fibres sometimes make contact with these processes and also with the cell bodies (↗). The radices n. hypoglossi are present at B. × [illegible]



FIG 9 Group B? Nialamid treated rat Transverse section A number of small to medium sized nerve cells with round to oval cell bodies and a strong yellow fluorescence are present mainly within the region of the nuc raphe obscurus just cranial to the level of the motor decussation The ventral part of the group is in the lower part of the figure × 100



FIG 10 Groups B1 and B3 Nialamide treated rat Transverse section A number of small to medium sized closely packed round to oval nerve cells of medium to strong yellow fluorescence belonging to group B1 can be seen surrounded by a large number of varicose intensely green fluorescent fibres within the rostral part of the nuc raphe pallidus (4) Dorsally to the pyramidal tracts (*B*) can be seen cells of the same size and form as in group B1 sending processes medially and laterally and sometimes even ventrally into the pyramids (↗) A small number of green fluorescent varicose fibres can also be seen within this group (B3) lying close to cell bodies and their processes (↗) The basal arteries (*C*) in the sulcus between the pyramids show a rich adrenergic innervation of the o

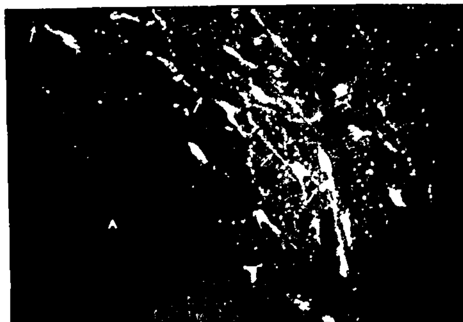


FIG. 11. Group B3. Nialamide treated rat. Transverse section. Lateral to the pyramidal tract (T) can be seen a number of medium sized oval and multipolar cells of medium to strong yellow fluorescence. Numerous fine yellow fluorescent processes (↗) extend in a medio-lateral direction enclosing the pyramidal tract. ×16.

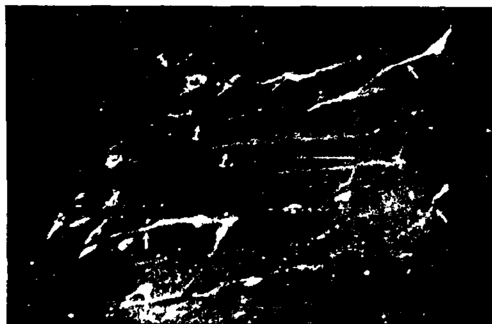
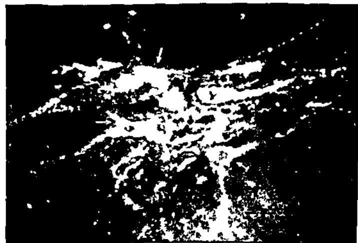


FIG. 12. Group B3. [illegible] treated rat. Transverse section. A number of medium sized oval and [illegible] cells of [illegible] yellow fluorescence can



Fig 13 Group A Normal rat Transverse section A group of small to medium sized round to oval and often clearly multipolar cells of medium to strong green fluorescence can be seen to accompany medially the outgoing fibres of the facial nerve (F) lying lateral and dorsal to the nucleus olivaris superior (B) partly within the tractus rubrospinalis (C) close to the ventral surface of the brain (/) A fold in the section is seen at D × 40



Fi 14 Group A5 Normal rat Transverse section The oval and multipolar green fl re cent cells were of both strong ( ) and medium (↗) intensity Fine varicose g n fluorescent fibres can be seen to be in close contact with the cell bodies (↗) ×0



FIG. 15 Group A. Normal rat. Transverse section. Within the locus coeruleus can be seen densely packed medium sized round to oval nerve cells of strong green fluorescence. The cell mass is present just under the lateral surface (↗) of the fourth ventricle (1). × 100

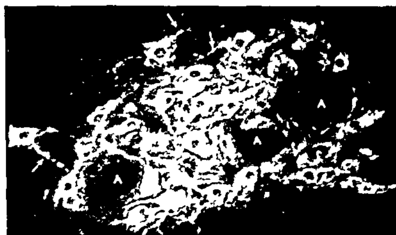


FIG. 16 Group AC. Normal rat. Transverse section through the periphery of the locus coeruleus. The processes ( ) of the strongly green fluorescent round to oval and often multipolar nerve cells lie fairly close together (↗). The green fluorescence in the cell bodies is accumulated in a zone around the non-fluorescent nucleus. Three small transversely cut vessels (A) are present among the cells. × [illegible]

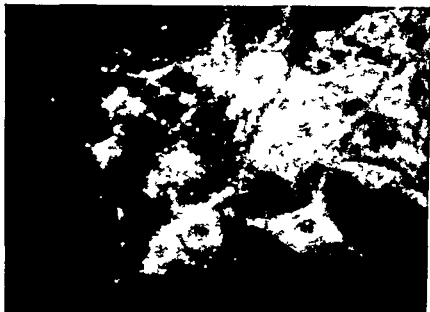


Fig 17 Group A6 Normal rat Transverse section Medium sized multipolar strongly green fluorescent nerve cells with an accumulation of fluorescence around the nucleus can be observed within the ventral part of the locus coeruleus × 460



Fig 18 Group A6 Normal rat Transverse section Three medium sized oval medium green fluorescent nerve cells from the ventral part of the locus coeruleus are observed Two of them have a distinct accumulation of fluorescence in a zone around the non fluorescent nucleus In one of the cells this zone (↗) has a dusty appearance A few fine varicose green fluorescent fibres can be seen in close contact with the cell body (↗) × 460



FIG. 10. Group A6. Normal rat. Sagittal section. The densely packed green fluorescent nerve cells of the locus coeruleus are seen at A (see Fig. 18). Adjacent to this area are localized the large round non-fluorescent nerve cells of the nuc. tractus mesencephalici trigemini (B). These cells are surrounded by fine varicose green fluorescent fibres. At C can be seen a group of oval and sometimes clearly multipolar strongly green fluorescent nerve cells which seem to be somewhat larger than those of the locus coeruleus. [illegible]0



FIG. 20 Group A7 Nialamid treated rat Transverse section A number of medium sized strongly green fluorescent nerve cells are scattered within the reticular formation ventral to the ventral portion of the pedunculus cerebellaris superior × 100

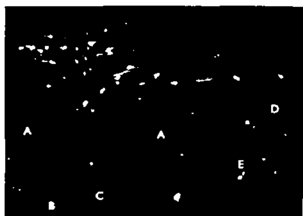


FIG. 21 Group B3 Reserpine–nialamide treated rat Transverse section A number of oval mainly medium sized strongly yellow fluorescent nerve cells are situated mainly among the fibres of the lemniscus medialis just dorsally of the trapezoid body (A) A few however are present laterally (D) of the trapezoid body and a few small cells ventrolaterally (E) just under the ventral surface the brain No adrenergic innervation is observed in the basal artery (C) 40



Fig. 22 Group B8. Reserpine-nialamide treated rat. Transverse section. A number of small to medium sized round to oval yellow fluorescent nerve cells of a medium intensity are found in the midline under the fourth ventricle (*A*) at the level of nuc. motorius n. trigemini. ×100

Fig. 23 Groups A8–A9. Normal rat. Transverse section cut approximately with the axis of König and Klippel. Unless otherwise stated this applies also to the other figures on the same plate. A fairly large number of small to medium sized round to oval nerve cells of medium green fluorescence are observed within the zona compacta (*A*) ventral to the lemniscus medialis (*D*) and several within the zona reticulata (*B*). These cells belong to group A9. Numerous cells of similar appearance are observed within the reticular formation (*C*) dorsal and dorsolateral to the lemniscus medialis (*D*). These cells belong to group A8. As seen in the micrograph there is no distinct borderline between groups A8 and A9. ×100

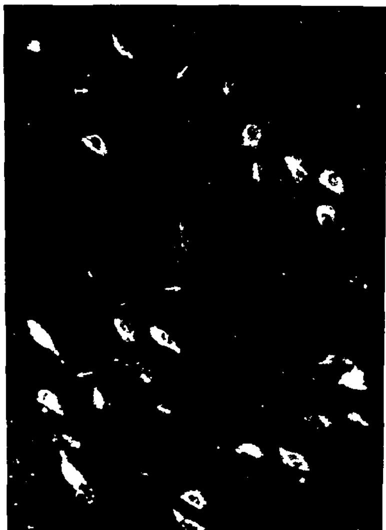


Fig. 4. Group A8. Normal rat. Transverse section. A number of mainly medium sized round to oval nerve cells of medium green fluorescence and with distinctly fluorescent processes (↗) can be seen within the reticular formation. Fine varicose green fluorescent fibres (↖) are present among the fluorescent cells. × 250

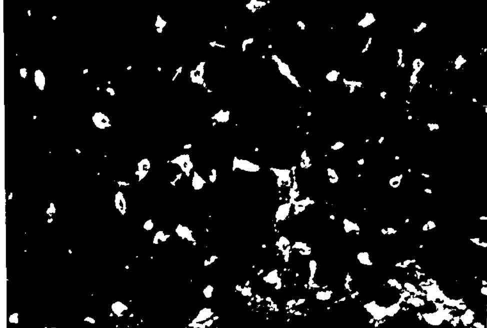


Fig. 25. Group A8. Nialamid treated rat. Transverse section. A large number of small to medium sized, round to oval nerve cells of medium green fluorescence are present within the lateral part of the reticular formation. A few are seen to be clearly multipolar (↗) and a few have a distinct accumulation of fluorescence in a zone around the nucleus (↗). ×10[illegible]



Fig. [illegible] Group A9 Normal rat Transverse section A large number of small to medium sized cells of medium green fluorescence are present within the zona compacta (*A*) ventral to the lemniscus medialis (*D*) and among the fibres of the n oculomotorii (*E*) It is impossible to determine in the microscope exactly when the latter cells cease to be in the zona compacta A number of cells are present within the zona reticulata (*B*) and a few belonging to group A8 within the reticular formation (*C*) ×40

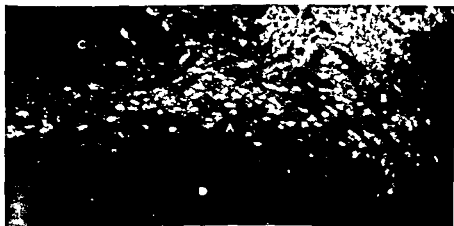


Fig. [illegible] Group A9 Normal rat Transverse section A large number of small to medium sized nerve cells of medium green fluorescence are present within the zona compacta (*A*) ventral to the lemniscus medialis (*C*) and among the fibres of the n oculomotorii (*B*) No fluorescent cells are present within the zona reticulata (*D*) ×100

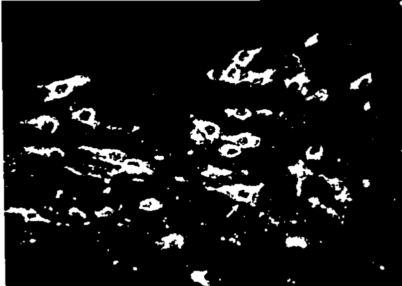


Fig 28 Group A9 Normal rat Transverse section Some small to medium sized round to oval nerve cells of medium green fluorescence are present within the zona compacta In a few of the cells (↗) a distinct fluorescent zone can be observed around the nucleus 50

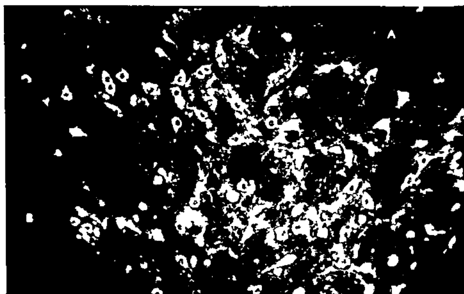


Fig 29 Groups A9 and A10 Normal rat Transverse section A large number of small to medium sized round to oval nerve cells of medium green fluorescence are present between the nu interpeduncularis (*B*) and the lemniscus medialis (*A*) in the borderzone between groups A9 and A10 and partly also among the radices n oculomotorii 16



Fig 30 Groups A9 and A10 Normal rat Transverse section A very large collection of small to medium sized round to oval nerve cells of medium to strong green fluorescence are seen to be present within the area ventralis tegmenti (*C*) and the zona compacta (*B*) ventrolateral to the lemniscus medialis (*A*) Medially it is impossible to decide when the fluorescent cells of the zona compacta cease to appear The cells within the medial part (*C–D*) of the area ventralis tegmenti probably belong to group A10 ×40

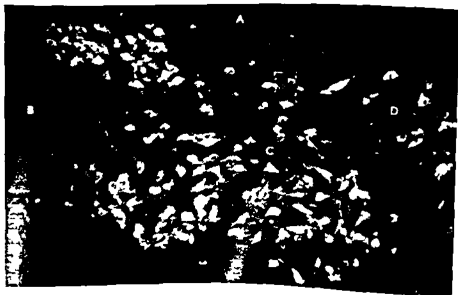


Fig 31 The nerve cells within the zona compacta and the area ventralis tegmenti in Fig 30 in higher magnification (For further explanation see text to Fig 30)

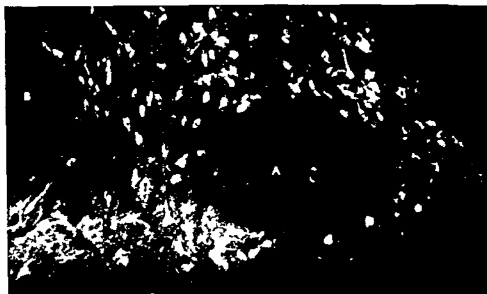


FIG 32. Groups A9–A10 Normal rat Transverse section cut approximately with the B–B1 axis of ZEMAN and INNES (1963) A large number of small to medium sized round to oval nerve cells of medium green fluorescence can be seen to surround the non fluorescent nuc tractus opticus basalis (A) with its tractus The lemniscus medialis is present at *B* × 100



FIG 33 Groups A9 and A10 Normal rat Transverse section (for the axis see text to Fig 32) A large number of small to medium sized round to oval nerve cells (A10) of medium green fluorescence are present just laterally and dorsolaterally of the nuc interpeduncularis (A) mainly within an area (*D*) corresponding to the nuc parabrachialis pigmentosus in the cat Many cells belonging to A9 are present within the zona compacta (*C*) ventral to the lemniscus medialis (*B*) in which a few fluorescent cells are present A number of fluorescent nerve cells are present dorsal to the nuc interpeduncularis within the rostral part of the caudal portion of the nuc linearis (*E*)



Fig 34 Group A10 Normal rat Transverse section A very large number of mainly small round nerve cells of medium green fluorescence are present dorsal to the cranial third of the nuc interpeduncularis (*A*) within an area (*C*) corresponding to the nuc parabrachealis pigmentosus in the cat and within the rostral part of the caudal portion of the nu linearis (*E*) Some cells (*F*) are observed in the border zone to A9 between the nuc interpeduncularis and the lemniscus medialis among the radices n oculomotorii The nuc ruber which appears completely free of fluorescent material is present at *D* ×40

Fig 35 Group A10 Normal rat Transverse section A very large number of mainly small and round nerve cells of a medium green fluorescence are present dorsal to the cranial third of the nuc interpeduncularis (*A*) within the area of the rostral part of the nuc lineari pars caudalis (*B*) and the area (*C*) corresponding to the nuc parabrachealis pigmentosus in the cat The dorso ventral direction is indicated by the arrow (←) ×100

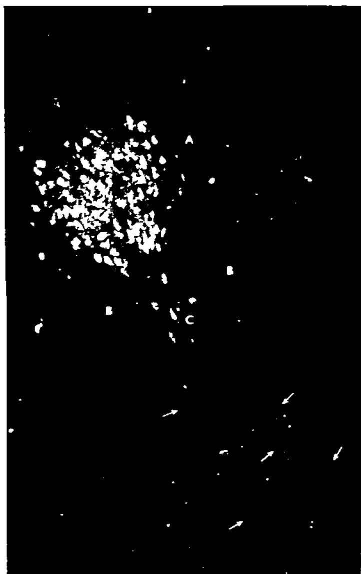


FIG. 36. Group I-7. Reserpine–nialamide treated rat. Transverse section. A closely packed mass of small to medium sized round to oval nerve cells of a medium to strong yellow fluorescence is present within the nuc. raphe dorsalis (A) dorso medial to the fasciculi longitudinales mediales (B). A large number of yellow fluorescent axons pass between the fasciculi longitudinales mediales (C) ventrally towards the nuc. interpeduncularis ( ). ×100



FIG 37 Group B8 Reserpine–nialamide treated rat Transverse section A large number of small to medium sized round to oval nerve cells of medium to strong fluorescence are present within the area of the nuc raphe medianus dorsal to the beginning of the nuc interpeduncularis (*A*) A number of perpendicularly cut yellow fluorescent axons are situated between the decussating fibres (↗) ×100

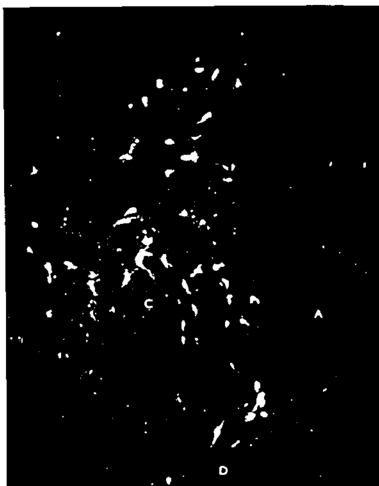


Fig. 38. Group B9. Reserpine–malamid treated rat. Transverse section. A large number of small to medium sized round to oval nerve cells of medium to strong yellow fluorescence are present within the lemniscus medialis (*C*) and ventrally of this close to the ventral surface of the brain (*D*) lateral to the nuc. interpeduncularis (*A*). A number of cells are present within the reticular formation (*B*). 16[illegible]

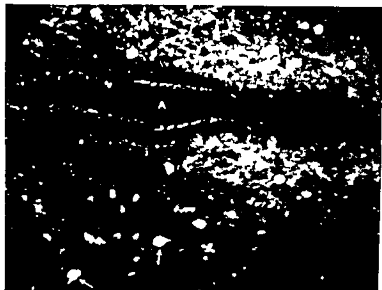


FIG 39 Group A1* α Methyl m tyrosin treatment (two doses of 400 mg/kg 15 and 13 h before killing) Transverse section (for the axis see text to Fig 32) A number of small round green fluorescent nerve cells are present within the area of the nuc arcuatus (*B*) and the ventral portion of the nuc periventricularis anterior (*C*) close to the third ventricle (*A*) The fluorescence is normal or somewhat increased (↗) ×165

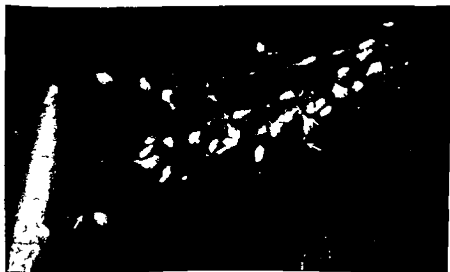


FIG 40 Group A9 α Methyl *m* tyrosin treatment (two doses of 400 mg/kg 18 and 16 h before killing) Transverse section A number of small to medium sized oval nerve cells often with distinct green fluorescent processes (↗) are present within the zona compacta Normal fluorescence intensity 165

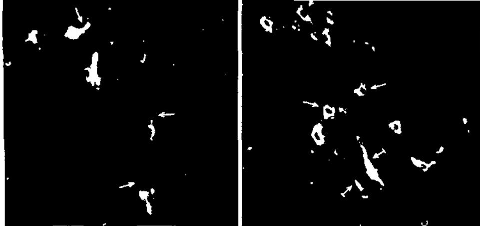


Fig 41 Group A1 Normal rat Transverse section The cells show a weak or medium fluorescence The fluorescent material is evenly distributed within the cytoplasm and present also in the processes (↗) Several fine varicose green fluorescent fibres are present ×165

Fig 42 Group A1 Reserpinized rat (10 mg/kg 6 h) Transverse section In most of the cells the zone of reappearing green fluorescent material around the nucleus is clearly seen being of medium fluorescence (↗) A few cells (↦) already have fluorescent processes ×165



Fig 43 Group A1 Reserpinized rat (10 mg/kg 8 h) Transverse section In some of the nerve cells a strong green fluorescence is present throughout the cell body which means that an above normal regeneration has occurred in the pericarya of these cells (↗) The fluorescent processes (↦) are seen with a fairly weak fluorescence and have not yet been filled up with green fluorescent material No varicose green fluorescent fibres are present ×165

Fig 44 Group A1 Reserpinized rat (10 mg/kg 12 h) Transverse section In most of the cells the entire cell body is filled up with fluorescent material of strong intensity Weakly fluorescent processes are also seen (↗) No varicose green fluorescent fibres are present ×165

Uppsala 1964. Almqvist & Wiksells Boktryckeri AB [illegible]

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Dynamic Response of Chest Wall and Lung Injuries in Rabbits Exposed to Air Shock Waves of Short Duration

BY

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STOCKHOLM 1964

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RESEARCH INSTITUTE OF NATIONAL DEFENCE SUNDBYBERG
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Abstract

Clemedson C J and A Jonsson *Dynamic response of chest wall and lung injuries in rabbits exposed to air shock waves of short duration* Acta physiol scand 1964 62 Suppl 233

The movements of the chest walls of rabbits exposed to steep-fronted air shock waves of short duration produced by 50 g spherical charges of TNT were recorded by means of a mechano-electric motion transducer originally developed for this investigation The main purpose has been to study the correlation between the deformations of the chest and the damage inflicted to the underlying organs especially the lungs The motion parameters amplitude velocity and acceleration of the chest wall were correlated to the physical characteristics of the shock wave as well as to the lung injury produced The lung injuries were found to be highly correlated to the impulse of the incident shock wave and also although to a lesser degree to the maximum overpressure of the shock wave and to the velocity and acceleration acquired by the chest wall The correlation of the lung injuries to the maximum amplitude of the inward displacement of the chest wall on the other hand was found to be much lower or even uncertain The following critical values of the motion parameters were established If the chest wall receives an impulse load of such a strength and duration that an inward relative movement results with a velocity of more than 15 m sec^{-1} attained within a period of time of 150—200 μs *i e* involving accelerations of the order of 10^4 *g* there is a high probability that a severe lung injury will result Corresponding maximum amplitudes of the displacement of the chest wall were 5 to 7 mm The lower limit of effective reflection pressures and impulses causing severe lung injuries were 10 bar and 1 5 gf sec cm^{-2} respectively The biomechanical events occurring in the exposed chest are discussed in some detail

It is by now well known that the primary effects of a blast exposure of a living organism *i e* the direct effects caused by the blast produced variations in environmental pressure are to be found mainly in air or gas containing organs such as the lungs gastrointestinal tract the ear and the paranasal sinuses (for references see *e g* Clemedson 1949 Clemedson 1956 White and Richmond 1959) Since the most conspicuous pathologico-anatomical changes in blast injury especially in air blast injury are lung injuries manifested as pulmonary haemorrhages and rupture of alveolar walls the mechanism causing these injuries is of very great interest

The origin of the lung injuries caused by shock wave exposure has been discussed by a number of authors but the opinions are diverging and the mechanism is still essentially unknown Generally it has been believed that the shock wave or pressure wave originating from the impact of the air shock wave on the body surface propagates through the body and in some way causes the lung

injuries In a number of publications from our laboratory (Clemedson and Pettersson 1956, Clemedson 1956 Clemedson and Jonsson 1960, 1961 a and b, 1962) it has been shown that a pressure pulse propagates through the body and also through the lungs when an animal is exposed to the shock wave from a detonating high explosive The pressure phenomena accompanying the exposure of a biological target to a shock wave have also been dealt with in some detail by White and Richmond (1959) Schardin (1950) has discussed the possibility of a spalling effect occurring at the fluid air interfaces in the lung alveoli and resulting in fragmentation haemorrhage and edema when a pressure pulse of sufficient magnitude propagates through the thorax When dealing with possible mechanisms of the lung blast injury, he also refers to the analogy of the implosion effects caused by a shock wave which passes through water containing small air bubbles The liquid surrounding the bubbles is accelerated and the bubbles are compressed At the surface of the bubble the energy of the pressure pulse is transformed into kinetic energy The volume of the bubble is highly reduced and very high local pressures occur In the strongly compressed bubble pressures of the order of magnitude of 10^5 bar may result, and from each bubble imploded in this way a strong shock wave originates Pressure recordings in the lungs by means of small piezoelectric pressure transducers have however failed to demonstrate pressure peaks that should have resulted from such locally generated shock waves (Clemedson and Pettersson 1956 Clemedson and Jonsson 1961 a 1962)

In earlier discussions of the mechanism of lung damage in blast exposure, the thoracic cage has often tacitly been considered as a rigid system in the sense that the importance of larger mass movements have been omitted For lack of quantitative information the forced motion of the chest wall or compression of the whole thorax has usually not been taken into consideration It is evident, however that such displacements occur and must play a role in the building up of the pressure pulses passing through the lungs and causing the damage They have also been verified experimentally by Clemedson and Criborn (1955) by means of a pencil line strain gauge recording method and by the same authors (unpublished experiments 1956) by means of a small accelerometer attached to a rib

White and Richmond (1959) state that an inward movement of the chest and abdominal walls is one of at least four possible mechanisms to account for and to have an influence on the development of pressure differentials across the chest wall and within the lungs and which may also account for the resulting lung damage The effects of a very fast inward movement of the chest wall in a blast exposed animal causing a kind of piston effect has been subjected to theoretical considerations and calculations of the forces involved by Penney and Bickley (1943 see Penney and Pike 1950) based on Zuckerman s (1940 1941) animal experiments but to our knowledge no experimental approach has been made earlier to clarify this very important question

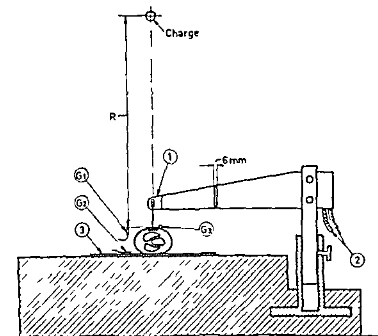


Fig 1 Schematic diagram of experimental arrangement 1 metal arm with perspex housing containing resistance coil of mechano-electric motion transducer 2 cables connecting transducer to cathode ray oscilloscope and to source of voltage 3 isolating rubber sheat on concrete basement G pressure gages G1 in free air at the same level as front chest wall of experimental animal G2 at supporting surface G3 on front chest wall of animal R distance from center of charge to front chest wall of rabbit

The present investigation therefore was undertaken with the aim of studying the dynamic response of the chest wall to the pressure impulse load of a high explosive shock wave and the correlation between the motion parameters of the chest wall *i e* the amplitude of relative movement the velocity and the acceleration and the lung injury produced

Methods

Sixty rabbits of 2 5 to 3 0 kg body weight were used for these experiments All animals were anesthetized with urethane administered intravenously in a marginal ear vein in a dose of about 1 5 g/kg wt

The animals were exposed to shock waves in the open air They were lying on the left side without any fixation on a horizontal steel plate fixed to a concrete base plate and with the right side facing the charge The animal was electrically isolated from the steel plate and the ground by means of a piece of rubber sheat 3 mm thick

As high explosive spherical charges of cast and turned TNT were used The charge was suspended over the animal exactly at a line through the center of the right chest wall and perpendicular to the steel plate The weight of charge was in all experiments 50 0 g The charge was ignited by means of a primer of 1 0 g of PETN (pentaerythritol

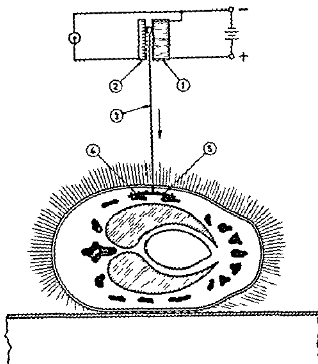


Fig 2 Schematic diagram showing mechano-electric motion transducer and fixation of its slider made of music wire to chest wall of animal 1 resistance coil of transducer 2 graded contact silver plate of transducer 3 slider 4 wire netting plate 5 fixation of wire netting plate to a rib

etranitrate) and an electric blasting cap containing 0 7 g TNT and 0 3 g lead azide and lead *trinitroresorcinate*

The pressure pattern of the incident shock wave was recorded by means of a lead zirconate titanate pressure transducer manufactured by Atlantic Research Corp Alexandria Va U S A The transducer type LC-10 used in this investigation is of the same shape and size as the transducer type BC-10 which has been extensively used in our laboratory earlier (see Clemedson and Pettersson 1956 Clemedson 1956 Clemedson Jonsson and Pettersson 1956 Clemedson and Jonsson 1961 a and c 1962) In most cases the pressure gage was fixed in the air close to the chest of the rabbit (less than 10 cm from the gage point used for the recording of the motion of the chest wall) and at the same level over the steel plate as the upper or front (right) surface of the chest (G1 in Fig 1) In some experiments the gage was placed at the steel plate close to the thorax of the animal (G 2 in Fig 1 In a few experiments the gage was located at the front side of the chest and as close to the above mentioned measuring point as possible without disturbing the recording of the movements of the chest wall (G 3 in Fig 1)

For the recording of the movements of the chest wall a recording method was developed which is based on the use of a potential divider with a very light and stiff slider firmly fixed to the object (see Figs 1 and 2) The recording of mass movements in biomechanical systems which are exposed to air shock waves makes great demands upon the transducer among other things due to the fact that it must function in a milieu in which there occur considerable discontinuous changes in air pressure and temperature as well as accelerations of the order of magnitude of up to tens of thousands of *g* Of

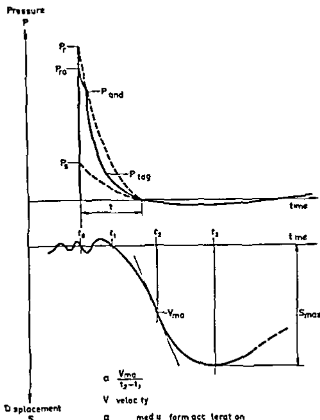


Fig 3 Schematic picture showing a pressure time diagram (upper curve) of the shock wave load and the corresponding displacement time response of the chest wall of a rabbit (lower curve) P is static overpressure and P maximum reflection overpressure at a plane rigid surface perpendicular to direction of propagation of shock wave P maximum reflection over pressure at thorax of a rabbit P d maximum overpressure when edge disturbances cannot be neglected P_{tag} stagnation pressure i e overpressure acting on a sol d rigid body in a uniform gas flow with a flow velocity equal to that of the shock wave t_+ duration of positive or overpressure phase t shock front reaches front chest wall of animal t_1 front chest wall starts moving inwards t maximum velocity (V_{max}) of inward movement of chest wall t time when maximum amplitude (S_{max}) of chest wall movement is attained

these parameters the transducer shall if possible reproduce only mass movement in one chosen direction Among methods used earlier for recording of movements of this kind strain gages capacitive transducers and piezo-electric accelerometers may be mentioned There kinds of transducers are however less suitable and often give results which are very difficult to interpret since it is very difficult to adapt them reliably to the object and since their masses often are too large in relation to the object to which they are attached Some of the methods used are also too sensitive to changes in ambient pressure

The transducer developed for the present investigation is especially suitable for the recording of transient nonperiodical translational motion It follows accurately the inward movement of the chest wall and this movement is reproduced as a displacement time diagram The amplitude of the movement of the chest wall is therefore obtained directly whereas its velocity and acceleration are deduced by means of one and two

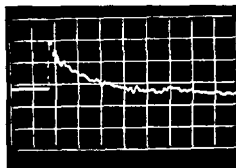


Fig 4 Pressure pattern of incident air shock wave Distance from charge 92 cm Time (between two vertical lines) 200 μs Peak static overpressure 1 2 bar

graphic differentiations of the displacement time curve respectively (cf Fig 3) The graphic differentiation method will of course introduce some errors but may be regarded as sufficiently accurate when dealing with biological material

In principle the transducer consists of a potential divider made of a flat resistance coil and a slider made of a music wire (3 in Fig 2) about 100 mm in length and 0 5 mm in diam. constituting the entire moving system which follows the mass movements to be recorded In the one end the wire has been flattened and bent to form a loop with a diameter of about 4 mm making a sliding contact which moves in the track between the finethreaded resistance coil (10 turns per mm) (1 in Fig 2) and a contact plate of silver also being the positive output point (2 in Fig 2) On the latter a scale for rough calibration is engraved The other end of the music wire is fixed to a small wire netting plate of stainless steel This plate which in this investigation had the dimensions 24 × 15 mm was firmly fixed directly to the ribs and musculature of the chest wall by means of surgical suture The skin was then replaced over the plate and closed by sutures over it This procedure gave a very high stability to the fixation of the plate to the chest wall The total weight of the plate and music wire used throughout this study was about 0 6 g i e about 0 17 g per cm² of the netting plate area Thus if only the mass of the chest wall (about 0 7 g cm⁻² coverad by the netting plate is considered the slider would increase that mass with as much as 20 per cent When dealing with air shock wave loads however much larger parts of the chest wall may be assumed to move equally In fact the whole part covering the lung area about 20 cm weight about 15 g) can be considered as a concentrated mass. In that case the mass load imposed by the slider being only about 4 per cent of the total mass. is negligible The main components of the transducer is enclosed in an inner transparent perspex housing protected by an outer metal casing (1 in Fig 1), which has been shaped to cause the least possible disturbance of the passing air shock wave The housing is fixed to the concrete base plate by means of a strong metal arm (see Fig 1) The arm can be raised and lowered by means of a gear rack and can be firmly locked in the right position This greatly facilitates the adjustment of the rabbit with the slider fixed to its chest wall to the main part of the transducer The voltage variations corresponding to the various positions of the contact part of the slider when it follows the movement of the chest wall impacted by the air shock wave is record[illegible] n an oscilloscope Tektronix type 555 with d c input.

The potential divider transducer is simple [illegible] in [illegible] te sensitivity of a transducer of this kind depends on the cl[illegible] s [illegible] as on the voltage fed to it In the transducer used in t[illegible] bout 6 volts was used which gave a sensitivity of a [illegible] The output impedance of the potential divider [illegible] is very low compared with the input [illegible] ec [illegible] The divider is, therefore pract [illegible] t

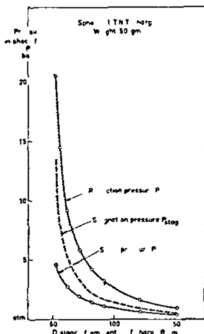


Fig 5 Pressure (P) in front of incident shock wave as a function of distance (R) from centre of charge For further explanation of legend see text

varies linearly with the motion of the slider the non linearity being less than 0 1 per cent Another advantage of the low output impedance is that interference pick up can easily be kept low so that long leads can be used without auxiliary equipment which of course is of importance in blast research Damping properties are very good and the fact that the transducer is insensitive to vibrations and pressure and temperature variations is of utmost importance in experiments of this kind The transducer is easily calibrated statically for example by means of a common caliper

The contact part of the slider has been adjusted so that the force necessary to overcome the friction is 20 to 30 gf This value is negligible as compared with the forces acting on the chests of the rabbits exposed to shock waves in this investigation The mechano-electric motion transducer and its function will be described more detailed elsewhere

The animals were killed exactly 30 min after the shock wave exposure by a rapid intravenous injection of 1 5 to 2 cc of Narkotal (sodium isopropyl 2 bromallyl N methyl malonylcarbamid) The lungs were carefully removed and weighed and the degree of lung injuries was determined according to the method introduced by Clemedson (1949) and expressed as the quotient of lung hemorrhage (L Q) This method has rather large errors amounting to 10 to 20 per cent but it has been found to be more accurate than a subjective estimation of the lung haemorrhages by ocular inspection only

Results

Physical characteristics of the shock wave load

Due to the high quality of the high explosive charges used in this investigation the pressure field around the detonating charge can be described by the well known scaling law (see *e g* Granstrom 1956) If the nature size and shape of

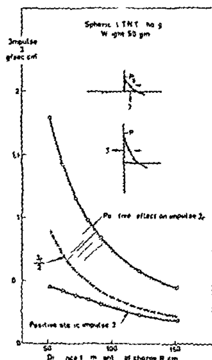


Fig 6 Impulse (I) as a function of distance (R) from centre of charge For further explanation of legend see text

the charge is known the characteristic properties of the air shock wave can easily be calculated A recording of the pressure pattern of the incident air shock wave at a distance of 92 cm from the charge is shown in Fig 4 A number of graphs showing some important characteristics of the incident air shock wave as a function of the distance from the charge are presented in Figs 5—7 Fig 5 shows the pressure (P) in the shock wave front as a function of the distance (R) from the charge P is the overpressure in the front of an undisturbed air shock wave and P_r the corresponding maximum overpressure when the shock wave is reflected against a plane rigid surface parallel to the front of the shock wave The curves are valid for the weight of charge (50 g) and distances used in this investigation The shock wave pressure acting on the animal (P_m) may be expected to be

$$P_s \leq P_m \leq P_r$$

which means that it will fall within the cross lined area in Fig 5 At parts of the body where the shock wave impacts at right angels P_m must be close to P whereas at parts approximately parallel to the direction of propagation of the shock wave it is more close to P All other parts where the impact of the shock wave is more or less oblique receive a pressure load which lies between these two extremes

Fig 6 is a graph showing the impulse (I) as a function of the distance from the charge I is the positive static impulse load on a plane surface parallel to the direction of propagation of the air shock wave and I is the reflection im

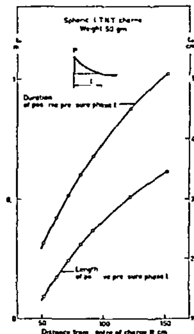


Fig 7 Duration t in ms and length l in cm of positive pressure phase of incident shock wave as a function of distance from centre of charge.

pulse received by the same plane rigid surface at right angels to the directio of propagation In accordance with the statement above about the pressur load the impulse load on the animal body (I_n) must be

$$I \leq I_n \leq I$$

According to Granstrom (1956) one might actually expect the real impuls load on an object of the kind used in this investigation to lie between I and I / (see Fig 6) The impulse load is dependent on the orientation of the impacte surface in the same manner as discussed above concerning the pressure bu one also has to consider the effects of edge disturbances Thus the impulse wil be greatest *i e* closest to I at the central parts of the chest wall which cover the main part of the lung

Fig 7 shows the duration (t_+) and "wave length (l_+) of the positive phas of the shock wave From this graph it is seen that at distances close to the charge the wave length is between 10 and 15 cm *i e* of the same order of magnitud as the lateral diameter of the chest of the experimental animal At the greates distance used the wave length is three to four times this value The significanc of the duration (t_+) of the positive pressure phase will be discussed later on

Dynamic response of chest wall to the shock wave load

The main qualities of the chest wall which determine its relative motion when it is affected by a shock wave are the mass of the chest wall the elastic ity of the ribs and intercostal tissues, and the resistance exerted by the under

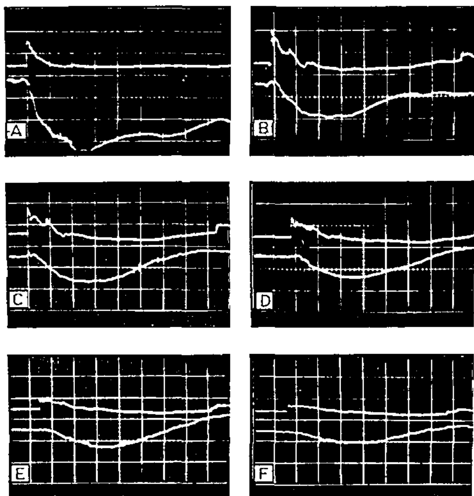


Fig 8 Recordings of incident air shock wave in gage point G1 in Fig 1 (upper curves) and inward movement of chest wall (lower curves) Distance from charge (R) A 57 B 72 C 82 D 92 E 122 and F 152 cm Time (between two vertical lines) 500 μs For shock pressure values see Fig 5 Pressure gage point in A G2 in B—F G1 Sensitivity of displacement transducer (lower curves) 2 2 mm between two horizontal lines in all recordings

lying tissues mainly the lungs and the heart Since it seems unlikely that the mass and elasticity of the chest wall can influence to any considerable degree the motion of the wall it is most likely that the retarding effect on the inward movement is exerted mainly by the underlying organs It is, therefore, justified to correlate the lung damage inflicted by the shock wave exposure to the movement of the chest wall The parameters which are of interest in this connection are the amplitude of the inward movement of the chest wall and its velocity and acceleration

An idealized diagram of the inward displacement of the chest wall in relation to the pressure time load is presented in Fig 3 The deflections in the displacement curve occurring in the time interval $t < t_0$ are probably due to disturbances

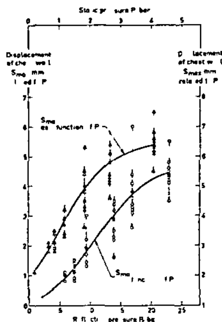


Fig 9 Maximum inward movement (S_{max}) of chest wall as a function of static pressure (P) and reflection overpressure (P) of incident shock wave

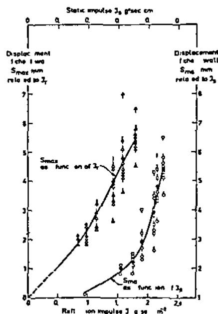


Fig 10 Maximum inward movement of chest wall as a function of static impulse (I) and reflection impulse (I) of incident shock wave

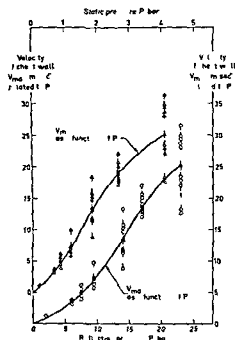


Fig 11 Maximum velocity (V_{max}) of chest wall as a function of static (P) and reflection (P) pressure of incident shock wave

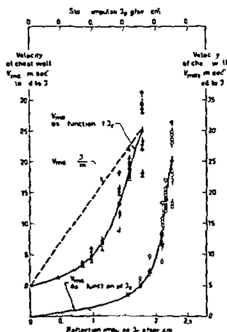


Fig 12 Maximum velocity (V_m) of chest wall as a function of static (I) and reflection (I_r) impulse of incident shock wave For explanation of curve $V_m = I/m$ see text

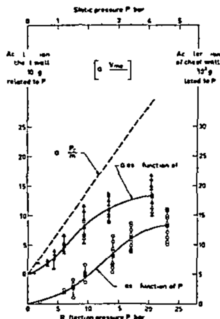


Fig 13 Acceleration (a) of chest wall as a function of static (P) and reflection (P) overpressure of incident shock wave The acceleration values on the ordinate axis shall be multiplied by 10³ For explanation of curve a = P /m see text

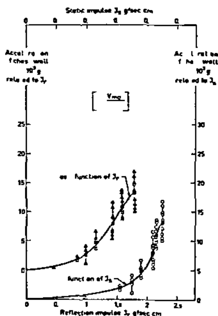


Fig 14 Acceleration (a) of chest wall as a function of static (I) and reflection (I) impulse of incident shock wave

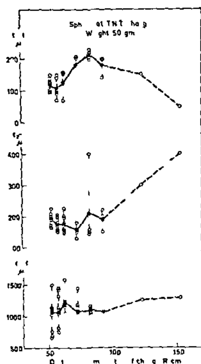


Fig 15 Relevant time intervals in μs of reponse period of chest wall as a function of the distance from centre of charge Time intervals are t_1—t_0 from arrival of shock front to chest wall to first indication of movement of chest wall t_2—t_1 beginning of movement to attainment of maximum velocity of chest wall and t_3—t_1 beginning of movement to attainment of maximum amplitude of inward movement of chest wall

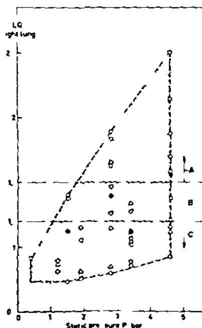


Fig 16 Degree of lung injury (quotient of lung hemorrhage) of front lung as a function of static overpressure of incident shock wave Hatched area comprises all exposed animals Area A severe B moderate and C slight or no lung injury

caused by the incident air shock wave when it passes over the metal arm with the transducer housing

Fig 8 shows some recordings of the motion of the chest wall in blast exposed rabbits at various distances from the charge. The upper curve in each recording is the pressure pattern of the incident shock wave and the lower curve shows the displacement or inward movement of the chest wall

Figs 9 and 10 show the recorded maximum displacement of the chest wall as a function of the static and reflection overpressure (Fig 9) and static and reflection impulse (Fig 10) of the incident shock wave In these curves as in the following the recorded value in each rabbit (open symbols) as well as the mean value for all animals at each distance used (black symbols) are presented Some of the points evidently show fairly large deviations from the corresponding mean value This is probably due to the fact that even minor irregularities of the pressure field as well as small variations in distance between charge and animal may be of great importance when such small charges are used as those in this investigation In Figs 11 and 12 the corresponding curves for the velocity of the chest wall are presented The dashed line in Fig 12 is a line drawn on the assumption that the force acting on the chest wall is I and that the whole impulse for some moment is taken up only by the chest wall Evidently the theoretical curve and that based on the recorded values tend to coincide at higher impulse loads This can be explained by the fact that the largest impulses are delivered to the chest in such a short time (see Fig 7) that the underlying parts have not received any appreciable part of the impulse during the time of loading The greater the distance between the two curves the larger is the part of the impulse that has been transmitted to the underlying tissues

Figs 13 and 14 show the order of magnitude of the accelerations of the chest wall as a function of static and reflection overpressure (Fig 13) and static and reflection impulse (Fig 14) The accelerations involved have been calculated by assuming that they are uniform during the period t —t_1 (*cf* Fig 3) Since it cannot be expected that the acceleration really is uniform during the whole period as the forces are not uniform the actual maximum accelerations probably have been larger than indicated by the curves in Fig 13 The dashed line in this Figure indicates the maximum possible acceleration if the force is P and the mass, m is that of the chest wall only

Fig 15 is a presentation of the three relevant time intervals of the response period of the chest wall (*cf* also Fig 3) These three intervals are 1 The time interval from the arrival of the shock front to chest wall (t_0) to the moment of the first indication of inward movement of the wall (t_1) 2 The interval from the beginning of the movement (t_1) to the moment when maximum velocity is attained (t_2) and 3 The interval from the beginning of the movement to the moment when the displacement of the chest wall has reached its maximum (t_3)

Displacement of chest wall and lung injury

In Figs 16—20 the degree of injury in the lung facing the charge as expressed

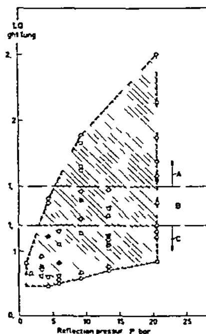


Fig 17 Degree of lung injury of front lung as a function of reflection overpressure of incident shock wave Legend see Fig 16

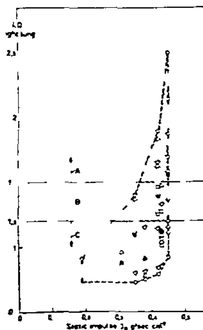


Fig 18 Degree of lung injury of front lung as a function of static impulse of incident shock wave Legend see Fig 16

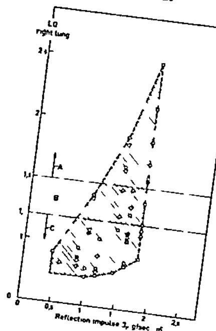


Fig 19 Degree of lung injury of front lung as a function of reflection impulse of incident shock wave Legend see Fig 16

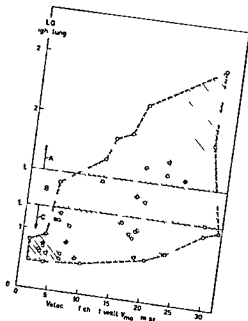


Fig 20 Degree of lung injury of front lung as a function of maximum velocity of chest wall Legend see Fig 16

by the quotient of lung hemorrhage has been related to the different loading characteristics of the air shock wave (Figs 16—19) as well as to the maximum velocity of the chest wall (Fig 20) The hatched area, which comprises all the quotients of lung hemorrhage of the exposed animals, has been divided into three parts Part A (quotients of lung hemorrhage > 1 5) comprises severely injured animals, part B (quotients 1 2—1 5) moderately injured, and part C (quotients < 1 2) slightly injured and noninjured animals The limits used have been defined and discussed in an earlier paper (Clemedson 1949) It is evident from Figs 16—20 that the lung injuries are highly correlated to the impulse of the air shock wave, and also rather highly correlated to the maximum pressure and to the acceleration and velocity imparted to the chest wall Between the displacement of the chest wall on the one hand, and the lung injuries on the other, the correlations were found to be much lower or even uncertain in this material

Discussion

When a human being or experimental animal is impacted and enclosed by an air shock wave, substantial elastic deformations of the body may result As far as can be judged from the open literature such shock wave produced deformations of biological material have earlier been subjected to theoretical considerations and experimental investigations only to a very limited extent (see Schardin 1950 White and Richmond 1959) The biomechanical processes which are connected with the origin of a blast injury generally seems to have been considered more or less from a pure acoustic point of view Without considering in more detail the mechanical deformation of the body as a system, the course of events seems to have been conceived as consisting of a number of transient elastic disturbances passing through the body with a velocity corresponding to the velocity of sound in water or about 1,500 m sec⁻¹ (see *e g* Corey 1946 Draeger Barr and Sager 1946 Schardin 1950 *cf* also White and Richmond 1959) Since the transmission velocity of such disturbances varies considerably in different parts of the body however it is evident that such a simplified treatment of the biomechanical events which occur in a living body impacted by a shock wave cannot be justified (Clemedson and Jonsson 1961 b and c, 1962) Thus it has been found that the velocity of a sound wave in compact bone is of the order of 3 500 m sec⁻¹ (Clemedson and Jonsson 1961 a) in muscular tissue about 1,580 m sec⁻¹ Ludwig 1950 Frucht 1953), in adipose tissue about 1,450 m sec⁻¹ (Frucht 1953) and in the lungs only of the order of 15—30 m sec⁻¹ (Clemedson and Jonsson 1962) That the deformations caused by the shock wave are of great importance in the biodynamics of blast injury is among others illustrated by the fact that the shock wave pressure that is necessary in order to produce blast injury is several times higher in an underwater detonation than when the exposure takes place in air (Corey 1946 Draeger, Barr and Sager 1946 Clemedson 1948) One possible explanation of this may be that the mass movements

are considerably smaller in water than in air at a definite pressure which means that a higher pressure is needed in water in order to cause a deformation which is large and fast enough to procedure blast injury

Since the most conspicuous pathological changes in blast injury are located in the lungs, the deformation of the chest would be of special interest Based on the experimental material collected by Zuckerman (1940, 1941), Penney and Bickley (see Penney and Pike 1950) made a theoretical investigation of the movements of the chest wall of some species of small laboratory animals exposed to high explosive air shock waves They arrived at the conclusion that the chest wall must acquire a certain relative velocity within a certain short period of time for a dangerous pressure pulse to be generated in the underlying lung tissue

In an earlier investigation (Clemedson and Criborn 1955) the occurrence of relative mass movements of the chest wall could be demonstrated in rabbits exposed to air shock waves but no numerical values of amplitudes velocities or accelerations could be given at that time In the present study an attempt has been made to determine these quantities experimentally By the method used it is possible to determine directly the amplitude of the movement but the prime goal has been to study the relative velocity of the chest wall imparted by shock wave loads of various strength By means of the recorded maximum velocities and the time it takes to reach these an estimation of the accelerations involved has also been made

Since shock wave produced pressure pulses in lungs are supposed to be responsible for the lung blast injury the building up of such pulses will be discussed in some detail here based on Fig 21 In this figure the thoracic system has been implified to a dynamic system showing some of the important physical characteristics of the chest It may of course be objectionable to divide such a complicated system as the chest into a few discrete parts having certain specified physical constants However when a pressure pulse passes through the chest from one side surface to the other it passes through a number of regions which from a dynamical point of view and for the kind of load used in this investigation may be considered as highly compressible and nearly incompressible respectively The chest wall heart and mediastinum are considered incompressible and these parts may be replaced by concentrated masses (a—b c—d and e—f in Fig 21) in the simplified mechanical system The highly compressible parts i e the lungs (b—c and d—e in Fig 21) may in this system be considered to have physical characteristics similar to those of an air spring in a kind of mass spring system

The main purpose of the present investigation was to study the relative motion of region a—b with respect to the underlying parts, and therefore pulses occurring in region b—c are of special interest The reason for that is as mentioned before that one may expect restoring forces exerted by the underlying lung during the inward movement of the chest wall to be of great importance

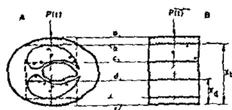
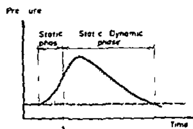



Fig 21 Schematic cross section of chest of rabbit with equidynamic system (upper figure) and idealized pressure pulse pattern in lung (lower figure) P(t) is pressure time load a—b front chest wall b—c front lung c—d heart and mediastinum d—e rear lung e—f rear chest wall X_b and X_d are independent variables necessary for description of dynamic response of chest system t_1 same as in Fig 3

and it may be assumed that the elasticity of the air confined in the lung (region b—c in Fig 21) to a high degree governs the motion of the chest wall (region a—b) during the dynamic response This elasticity is a continuous but for the kind of load used non linear function of the displacement of region a—b Thus, the elastic spring coefficient of region b—c in the equidynamic system will increase with increasing amplitude of the inward displacement of region a—b Consequently the simplified mechanical system must be a non linear one and it should appropriately have two degrees of freedom *i e* two independent variables X_b and X_d are necessary to describe the dynamic response of the system In this investigation the variable X_d can however, often be considered as constant during the most interesting part of the dynamic period, and the chest therefore, be looked upon as a simple non linear mechanical system with one degree of freedom As this system is a model of the thorax of a living animal one must also take into consideration that the elasticity of region b—c is continuously varying with time due to the changes in the volume of the heart which causes volume changes in region c—d and to the respiration which causes changes of volume in regions b—c and d—e For the sake of simplicity these effects however are omitted in this paper

A pressure pulse in region b—c may be built up differently depending on the characteristics of the load If for instance the load is an extremely short pulse of a high pressure but with a very small impulse value, then the transmission of the energy of the original shock wave to region b—c may largely be in the form of a longitudinal pressure pulse passing through region a—b as a change of condition similar to that occurring in a sound wave and causing a similar type of

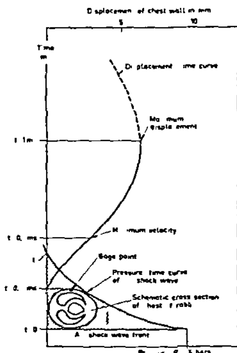


Fig 22 Typical dynamic response of chest wall of rabbit exposed to a steep-fronted air shock wave of short duration Displacement time curve corresponding to moderate or severe lung injury t₊ is duration of overpressure phase For other times see Fig 3

wave in region b—c For an ordinary air shock wave load this effect might to a large extent be attributed to the action of the steep air shock front and consequently the contribution of these sound like waves to the final pressure pulse in region b—c may be expected to be especially pronounced in the front of this pulse This is seen from the pressure pulse diagram in Fig 21 in which the period during which the sound like waves dominate is denoted the static phase It should be mentioned that the pressure loads used in this investigation have been to small to increase the density in region b—c enough for real shock fronts to develop in this region

Pulses of high pressure and extremely short duration are however mostly only of theoretical interest Usually, one has to consider the additional effects caused by the impulse of the air shock wave which causes an inward displacement of region a—b with respect to the underlying parts of the system and a proportional compression of region b—c Such a compression may be approximately described by the well known adiabatic law $P V^{\gamma} = \text{const}$, where P is the pressure V the volume and γ the ratio between the specific heats of air of constant pressure and constant volume respectively If however this compression takes place very rapidly *i e* if the region a—b is forced inwards with an acceleration and a velocity large enough to create a kind of shock wave in region b—c the condition in the front of this shock wave can be described by the Rankine Hugoniot equations (see *e g* Penny and Pike 1950)

$$E_1 - E_0 = 1/2\,(p_0 + p_1)\,(v_0 - v_1), \tag{1}$$
$$U = v_0\,[(p_1 - p_0)/(v_0 - v_1)]^{1/2}, \text{ and}$$
$$W = [(p - p_0)\,(v_0 - v_1)]^{1/2},$$

where $E_1 - E_0$ is the change in internal energy per unit mass, p_0 and p_1 the pressures and v_0 and v_1 the volumes of unit mass in the medium just in front of and behind the shock front respectively, U the velocity of the shock front and W the mass velocity behind the shock front. The dynamic period during which the adiabatic compression and the shock wave like waves contribute to the final pulse in region b—c has been denoted the static dynamic phase in Fig. 21. The name static dynamic is justified because sound like waves may exist also during the dynamic period.

It is evident from the present investigation that, in case of exposure to an air shock wave with a steep front, it takes 100 to 200 μs after the arrival of the front of the shock wave to the surface of the front side of the thorax (the time interval $t_0 - t_1$ according to Fig. 3) before any appreciable movement of the chest wall takes place. Practically this means that an object of the size of a rabbit will be entirely enclosed by the air shock wave before the chest wall starts moving (see Fig. 22). During this period, $t_0 - t_1$, the chest wall can be regarded as a rigid system and the events occurring in the body during this period are acoustic in nature, *i. e.* they are vibrations with small amplitudes, which can be described by a theory using constants such as density, ϱ and sound velocity c within uniformal distributed masses. After this period deformations of such a magnitude occur that a dynamic or acoustic dynamic theory, that is a theory dealing with masses and elastic spring forces, is more adequate. Now the chest wall is no longer a rigid system but can schematically be regarded as the above mentioned mass spring system. The chest wall starts moving inwards like a piston and a pressure pulse is created which passes through the underlying compressible parts.

In accordance with the theory mentioned above the passage of a shock wave generated pressure pulse through the lungs can be considered to be composed of one static and one dynamic phase. The static phase is predominant during the first part of the shock wave loading, *i. e.* before any significant deformations have occurred. During the later parts of the event, the dynamic phase is dominating, and it is in all probability this phase which is of crucial importance for the production of the pulmonary injuries in blast. Of course such a division of the event into two phases is at the present stage to be considered as a pure hypothetical approach. In the real pressure pulse propagating through the thorax after the impact of a shock wave it will hardly be possible to distinguish between the static and the dynamic phase.

The physical properties of the shock wave that are of interest from the bio mechanical point of view are first of all the steepness of the front, the pressure in this front (P and P in Fig. 5), the duration, t_+, of the positive pressure phase and the impulse (I and I in Fig. 6).

Concerning the steepness of the shock front it is sufficient to mention that the rise time to peak pressure is faster than the response time of the exposed structures and it can be estimated to be of the order of 10^{7} to 10^{8} sec Since the charges used in these experiments were small, the durations t of the positive phase are also small (see Fig 7), and the time during which the animal is enclosed in the positive pressure field is short This was an advantage in the present investigation where the primary goal was to study the inward movements of the front chest wall and to try to find the relations between these movements and the damage inflicted to the lungs and other underlying organs It can be assumed in this case that the displacement of the chest wall is to the greatest part determined by the maximum overpressure and the positive impulse of the shock wave (*cf* Figs 5 and 6) Figs 5 and 6 show that close to the charge *i e* within the most interesting area from the point of view of biological damage the reflection pressure P and reflection impulse I respectively are 3 to 4 times greater than the static or sideon pressure *P* and impulse *I* respectively

It should be pointed out here that due to the great difficulties in determining the pressures and impulses acting on an object of the irregular and flexible kind as an animal body, all pressure and impulse values used in this investigation have been calculated by using the well known scaling laws valid for spherical charges of TNT These values have been obtained from Granstrom (1956) P can be assumed to have an accuracy of about ± 5 per cent, I about ± 10 per cent and P and I about ± 20 per cent Furthermore the load conditions in this treatment have been idealized so that P to take one example means the reflection overpressure at a plane rigid surface perpendicular to the direction of propagation of the shock wave In the same way I is the impulse which is received by such a surface assuming that no edge disturbances will reach the measuring point during the time interval $t_0 — t_+$ and furthermore that the wave is totally reflected These definitions apply strictly only to an one dimensional propagation of the air shock wave In spherical propagation the events will be more complicated Furthermore to determine the effective pressures and impulses which act on the body surface of the animal is exceedingly complicated and since also other uncertainties of biological experiments of this kind are rather great it has not been found necessary to differentiate in this treatise between one dimensional and three dimensional propagation Some experiments have however been made earlier by Clemedson and Jonsson (Clemedson 1959) to compare the pressure time events at the body surface of a rabbit with those at a plane rigid surface In these experiments a small cylindrical pressure transducer was employed and the shock wave pressures used were within the interval 0 5—1 2 bar of static overpressure which corresponds to 1 2—3 5 bar of reflection overpressure It was found that the pressure at the animal body generally was about 10 per cent lower than at the rigid surface This value however is very uncertain and it is highly dependent among others on the physical nature of the tissues and structures below the surface

Determining for the course of the shock pressure load, in addition to the physical properties of the air shock wave is, as mentioned earlier, also the external and internal physical properties of the exposed object. Among external properties of importance, the size, shape, surface characteristics and orientation with respect to the direction of propagation of the shock wave are some of the most important ones. The internal properties will be dealt with more in detail later on.

Fig. 3 gives a qualitative conception of the course of the shock wave load on an object of finite dimensions in which edge disturbances cannot be neglected. It is assumed in this figure that the pressure acting on those parts of the object which are perpendicular to the direction of propagation is P_r or a little less (P_* in Fig. 3). The pressure acting on parts parallel to the direction of propagation is P and all other parts are supposed to be exposed to pressures corresponding to oblique reflection pressures, *i. e.* pressure values being between P and P_r. Fig. 3 is intended especially to illustrate the pressure time events occurring at a part of the body which is essentially perpendicular to the incident shock wave, and it will therefore fairly well illustrate the course of events at those parts of the chest wall that are of interest in this study.

At the moment when the front of the air shock wave arrives at the front surface of the exposed object the pressure in the shock front rises instantaneously from P to P or P_* and the pressure time course will then go on as if the surface had infinite dimensions until the pressure has decreased to the value $P_{r,ed}$ which is the pressure acting at the moment when the edge disturbances have just reached the measuring point. At this moment a very complicated course of unloading starts which goes on until the pressure has reached the value $P_{t,g}$. Actually P_{tag} is the pressure that is acting on a solid rigid body in a uniform gas flow having a flow velocity equal to that of the shock wave. According to Granstrom (1956) the stagnation pressure $P_{t,g}$ for a solid body of finite dimensions is 0.5 to 0.6 times P_r. This applies for static pressures up to 4 bar. It can therefore be assumed that the effective impulse which acts on the chest wall of the front side of the animal will be somewhere between I and I /2. At the central part of the chest wall, *i. e.* the part closest to the charge it is probably closer to I.

In accordance with the earlier discussions of the propagation of pressure pulses in a living body (*cf.* also Clemedson and Jonsson 1961 b and c, 1962) there is reason to treat the propagation from acoustic as well as from dynamic points of view. An acoustic theory would be particularly suited for the propagation through those parts of the body which have a very low compressibility whereas a completing non linear, dynamic theory is necessary for the description of how pressure pulses originate in highly compressible parts, *e. g.* in the lungs.

For the theoretical treatment from a biophysical point of view of the reaction of the organism to a shock wave load a number of constants are of great importance. In order to characterize the reactions during the first 100 to 200 μs after the arrival of the shock wave front to the chest wall, *i. e.* during the aforemen

tioned static phase (see Fig 21) before any mass movements of importance have taken place the density of the tissue ϱ and the velocity of sound, c are of importance since the propagation of waves of an acoustic type in a medium is dependent of the acoustic impedance ϱ c, of the medium Of special interest is the energy transition in the boundary layer between two media (*e g* between a—b and b—c in Fig 21) which differ greatly as to density and sound velocity *e g* the chest wall and the lung Since the density is for bone tissue $\cong$ 2 g cm^{-3} for muscle tissue $\cong$ 1 g cm^{-3} and for lung tissue only 0 25—0 5 g cm^{-3} and the velocity of sound in these tissues is 2 500—3 500 m sec^{-1} (Clemedson and Jonsson 1961 a) $\cong$ 1 500 m sec^{-1} (Frucht 1953) and 15—50 m sec^{-1} respectively the acoustic impedance of the chest wall thus is in round numbers 100—300 times greater than that of the lung This means that only a very small fraction of a pressure pulse which has been transmitted through the chest wall to the surface of the lung as an acoustic compression wave will pass over to the lung at each reflection at the boundary layer

When pressure pulses of this kind pass a boundary surface between two media with different acoustic impedances pressure and particle velocity must be equal on the two sides of the boundary layer These considerations give the following equations for the reflection overpressure P_{re} and the transmitted pressure P_t for example at the boundary between the chest wall and the lungs

$$P_{re} = \frac{\varrho_l c_l - \varrho_w c_w}{\varrho_l c_l + \varrho_w c_w} P_i \qquad (1)$$

and

$$P_t = \frac{2 \varrho_l c_l}{\varrho_l c_l + \varrho_w c_w} P \qquad (2)$$

where P_i is the pressure which is transmitted through the chest wall ϱ_l and c_l are density and velocity of sound respectively for the lung and ϱ_w and c_w the corresponding values for the chest wall When the first reflection takes place P is probably equal to P since the chest wall is too thin to be able to influence and change the pressure pulse to any degree worth mentioning From equation (2) it is evident that only a few per cent of the incident pressure P is transmitted to the lung at each reflection at the boundary layer If the density of the lung ϱ_l is taken to be 0 5 g cm^{-3} and the velocity of sound c_l is 50 m sec^{-1} and the corresponding values for the chest wall is $\varrho_t = 1$ g cm^{-3} and $c_t =$ = 1 500 m sec^{-1} P_{tr} in each reflection is found to be $\cong$ 0 033 P or about 3 per cent of P This fact indicates that the deformation of the chest wall in its entirety must be of great importance, when the chest is struck by a pressure pulse strong enough to damage the lungs

Among factors which are of importance for the dynamic response of the chest during the dynamic phase may primarily be mentioned the mass of the chest wall elasticity of the lung and natural frequency of the chest Thus, it is essentially the mass of the chest wall and the elasticity of the ribs which can to some

extent unload the underlying organs. At the magnitude of the shock wave load used in the present study, i. e. P_r up to 20 bar and t_+ about 0.5 ms, it does not seem very likely that either the mass of the chest wall, which was found to be about 0.7 g cm^{-2}, or the elasticity of the ribs could to any greater extent influence the pressure pattern in the lungs (*cf.* Penney and Bickley 1943). The lungs, therefore, are likely to be compressed by the inward movement of the chest wall. Since this compression can be assumed to be adiabatic, as there is not enough time for any appreciable flow of air or heat to occur during the compression of the chest, a pressure pulse of the kind previously discussed will pass through the lungs. The characteristics of this pressure pulse are related to the amplitude of displacement, velocity and acceleration of the chest wall, and if these quantities are known, it would be possible to estimate the compression conditions in the subjacent lung and also the pressure pattern.

In this connection it deserves mentioning, however, that it cannot under all circumstances simply be assumed that an one dimensional shock wave load applied to the surface of the chest will be decisive for the lung damage. Furthermore, one must be careful in estimating the compression of the lungs from the recorded movements of the chest wall. Part of the impulse received can, during the course of the load, have had time to propagate through one lung and may already have reached underlying parts (*e. g.* regions b—c and c—d in Fig. 21) before the load is over. This means that the "reference level" in the animal body to which the relative movements of the chest wall can be referred will vary with different durations of the load. In the cases of the greatest shock wave loads used in the present investigation, the time of application was so short that the pulse probably had not enough time to pass through the entire lung of the front side. That means that X_d in Fig. 21 may be assumed to be constant during the period of loading. This is also to a certain extent illustrated by Fig. 12 which in addition to the curves of the determined relationships between the velocity of the chest wall and the reflection impulse I, also presents a curve corresponding to the equation

$$\int_{t_0}^{t} P\,dt = m\,v - m\,v_0$$

where $t - t_0$ is the duration of the overpressure phase, P the reflection overpressure as defined earlier, m the mass of the chest wall per cm^2 and v and v_0 the velocity of the chest wall ($v_0 = 0$). The constructed curve

$$v = \frac{\int_{t_0}^{t} Pr\,dt}{m} = \frac{Ir}{m}$$

indicates that at the distances (R) closest to the charge, the chest wall will receive the whole impulse during the time of the shock wave load. In such a case it would be possible to determine more accurately the compression event in the lung from the movements of the chest wall.

It seems appropriate in this connection also to discuss the possible influences of the wave reflected at the hard surface under the animal on the recorded movements. The maximum velocity and the calculated accelerations will probably not to any greater extent be influenced by the reflected wave. From Fig. 15 it is seen that the maximum velocity occurs at about 200 μs after the moment when the chest wall started moving, i.e. at maximum 400 μs after the arrival of the shock wave front to the front surface of the animal. Thus the maximum velocity will occur at about the same moment as the air shock wave has engulfed the animal and been reflected back over it again. The pressure at the front surface of the animal is, however, much less in the reflected than in the incident shock wave. Actually, the pressure in the reflected wave is considerably less than the static pressure in the incident wave. Furthermore, there is a time interval of about 200 μs between the arrival of the pressure pulse to the chest wall and the starting of any movements of it, which are of importance for the recording. This speaks for the assumption that the velocity and acceleration values are relatively uninfluenced by the reflections against the supporting surface. The influence on the maximum displacement may be greater since these amplitudes do not occur until 1 to 1.5 ms after the movement has started. The influence of the reflected wave must, however, be relatively unimportant as compared with that of the incident wave. It is also very unlikely that the animal body as a whole will be able to move and thus influence the recording of the amplitudes during the time it takes for the chest wall to be displaced.

Another factor which is of great importance in a dynamical event of this type is the natural frequency of the thoracic system. Clemedson and Pettersson (1956) have recorded pressure pulsations in the thoraces of rabbits exposed to air shock waves. The natural frequency of the thoracic system was found to be about 250 cycles sec^{-1}. The present investigation shows that the chest wall of a rabbit under a shock wave load with a duration of about 0.5 ms and P up to 4 bar reaches its maximum displacement at between 1 and 1.5 ms after beginning of the displacement. It is, of course, very difficult to define natural frequencies for such a complicated system as the chest of an animal, but once again considering the system as a simple non-linear one with one degree of freedom, the following will apply. The effective natural frequency of such a system is dependent among other things also of the amplitude of displacement. Thus for very small values of impulses and displacements, the recorded values of t_d ($t_3—t_1$ in Fig. 15) may be equal to 1/4 of T_0 if T_0 is the natural period of the system. The time t_d will, however, decrease as the impulse increases and it may become considerably less than 1/4 of T_0, say 1/5 to 1/6 or even less. At present, it would therefore be reasonable to state that natural periods of the chest walls of rabbits exposed to shock wave loads of the kind used in this investigation are about 4 to 6 ms, corresponding to a natural frequency of 150 to 250 cycles sec^{-1}.

The natural period T_0 of the chest wall and the duration of the air shock wave t_+ will determine whether the effect of the shock wave load will be de-

pendent essentially on the pressure or on the impulse. If a dynamical system at rest is influenced by a pressure-time event having a positive duration, t_d, which is considerably shorter than the natural period, T_0, of the system, the load can be considered to be of impulse type and the pressure-time pattern is of secondary importance. If, on the other hand, t_+ is considerably greater than t_d, the load is of pressure type and the pressure-time pattern will be of greater importance. In the present investigation, T_0 was 4 to 6 ms and t_d ranged from about 300 μs to just over 1 ms. Thus, the shock wave load on the animal has been of the impulse type and the initial value of the velocity of the chest wall depends only on the momentum transmitted. For the sake of completeness, the recorded values have, however, also been related to the reflection pressures as well as to the reflection impulses. Furthermore, they have also been related to the actual static pressures and impulse, since these are easier to determine than the reflection values and therefore are more accurate. Since the load used was of impulse type, it is quite natural that the lung injuries should be most strongly correlated to the impulse of the shock wave. Of special interest is that only a very low correlation could be demonstrated to exist between the displacement amplitude of the chest wall and the lung injury.

The present investigation has shown, however, that a severe lung injury in a rabbit is produced only if the recorded maximum velocity of the chest wall is greater than 12—15 m sec^{-1}. It seems justified to conclude, therefore, that relative velocities of the chest wall greater than 15 m sec^{-1} attained within a period of time of 150 to 200 μs, i. e. involving accelerations of the order of 10^4 g, are very critical.

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Parathyroid Effect on Intestinal Absorption of Calcium and on Secretion of Calcium with the Digestive Juices in Vitamin D-Deficient Rats

BY

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Summary

The use of the metabolic balance technique in which net absorption of calcium is estimated has led to both positive and negative results regarding an effect of the parathyroids on intestinal calcium absorption Parathyroidectomy may by lowering the serum calcium affect the amount of calcium secreted with the digestive juices and therefore the net absorption of calcium In the work here presented true absorption was studied with the aid of radio calcium in rats con tinuously ingesting the isotope thus allowing estimation of digestive juice calcium and the coefficient of true absorption (absorbed fraction of diet calcium and digestive juice calcium) Ten vitamin D deficient adult rats were studied over 2 or more periods of 3 days each before and after parathyroidectomy (stages 1 and 2 respectively) and after injections of parathyroid extract (375—1100 units once daily or every 3 days) (stage 3) The mean serum calcium levels were approxi mately 9 and 5 mg/100 ml during stages 1 and 2 respectively and ranged between 9 and 13 mg/100 ml during stage 3 Six to 12 days after parathyroidectomy the coefficient of true absorption was re duced by 28 per cent and the digestive juice calcium by approximately 50 per cent The calcium intake did not vary significantly and the net absorption was the same as before the operation During stage 3 the digestive juice calcium was doubled while the results for true absorption were uncertain Digestive juice calcium was positively correlated with food consumption and serum calcium at all 3 stages It is concluded that a) parathyroidectomy reduces true absorption of calcium in vitamin D deficient rats and b) measurements of net absorption do not provide a reliable estimate of the absorptive ability of the intestine when the digestive juice calcium varies

Until a few years ago, the hypothesis of parathyroid stimulation of absorption of calcium from the intestine was based on scant indirect evidence, such as the low fecal calcium excretion in the majority of hyperparathyroid patients (Aub, Tibbetts and McLean 1937 and Robertson 1942) The early literature has been reviewed by Kenny (1961) Recently however, several reports have appeared presenting more direct evidence for this hypothesis (Rasmussen 1959, Cramer, Suiker and Copp 1961, Cramer 1963 Schachter 1963 and Kodicek *et al* 1964) Different techniques in different animal species both *in vivo* and *in vitro* have been employed in these studies and also in those failing to demonstrate an effect of parathyroid hormone on calcium absorption (Wasserman and Comar 1961 and Gran 1960 b) Of the different techniques the metabolic balance with calculation of net absorption would seem to provide a good estimate of the over all efficiency of the intestine in absorbing calcium under physiological conditions However in two studies in rats this technique has led to apparently conflicting results In one, injection of parathyroid extract appeared to increase net absorption of calcium (Cramer *et al* 1961), while in the other (Gran 1960 b) it was unaffected by parathyroid ectomy of vitamin D deficient animals The explanation for the discrepancy could be that when serum calcium changed, parallel changes in the concentration of calcium in the digestive juices influenced net absorption so that it only partly reflected changes in true absorption Consequently it was decided to study the para thyroid effect on intestinal absorption by measuring the balance of ^{45}Ca as well as total calcium in rats continuously fed a ^{45}Ca containing diet (Gran 1960 a) With the aid of this method the coefficient of true absorption (λ) can be calculated According to Nicolaysen Feg Larsen and Malm (1953)

$$\lambda = \frac{Ca_i + Ca_s - Ca_f}{Ca_i + Ca_s} \qquad \text{(Eq 1)}$$

where Ca_i is calcium intake Ca_s is digestive juice calcium and Ca_f is calcium in the feces

Interest has recently been aroused in the relationship between this effect of the hormone and that of vitamin D, the primary stimulant of calcium absorption According to Dowdle Schachter and Schenker (1960) the postulated parathyroid effect on the intestine may depend on the presence of vitamin D as has been claimed for the calcium

mobilizing action of parathyroid hormone (Harrison, Harrison and Park 1958) Since a preliminary experiment did not reveal such a relationship with regard to serum calcium regulation by the para thyroids it was assumed that neither was vitamin D required for the postulated intestinal effect of the hormone In fact, it was felt that the latter effect would be easier to demonstrate if the absorptive mechanism was not under the influence of vitamin D at all It was consequently decided to study vitamin D deficient rats before and after parathyroidectomy (stages 1 and 2) and also after high doses of parathyroid extract (stage 3) A preliminary account of the results was given at a symposium at Cornell University in May 1962 (Tove rud 1963)[1]

Materials and methods

Rats

Adult female rats were taken from this Institute s colony of interbred strains of white and hooded rats The colony has been maintained for over 20 years on diets containing minimal amounts of vitamin D At the time of the first balance period the rats were 14 months old and the body weights ranged from 170 g to 270 g The weights were relatively constant during the experiment

Diets

For 7 months prior to the first balance period the rats were given a stock diet lacking only vitamin D and containing approximately 0 5 % calcium and 0 5 % phosphorus (Toverud 1964) Carrots or spinach as sources of vitamin A were the only supplements to the diet The experimental diet was a purified vitamin D free high protein diet (Toverud 1964) It contained approximately 31 % protein from casein egg albumin and wheat flour, and the same amounts of calcium and phosphorus as the stock diet To the diet was added 0 5 % chromic oxide as an unabsorbable marker (Gran 1960 a) Determination of chromic oxide in the feces collected over a defined period of time allows accurate calculation of the food intake for that period This method was used successfully by Gran (1960 a) and was therefore routinely employed in the present experi

[1] In this reference some of the results in Table A were miscalculated Correct results are found in the present Table II

ment To one portion of one batch of the diet was added an aqueous solution of $^{45}CaCl_2$ This gave a specific activity in the diet of 575 counts per min per mg calcium In order to attain even distribution of the isotope it was necessary to add the following step to the procedure outlined by Gran (1960 a) Clumps formed after the addition of the isotope solution to the diet were dried ground in a mortar and re mixed with the diet in a machine Several random samples taken from the diet container indicated that the isotope was evenly

Table I Composition of experimental diet

Mean (± standard deviation) content based on analyses of 8 samples taken at the beginning of the experiment from the diet container (I) and at the end of the experiment from different food cups and the diet container (II)

	Ca per cent	P per cent	Cr_2O_3 per cent	^{45}Ca counts/min/g	$^{45}Ca/Ca$ counts/min/mg	Ca/Cr_2O_3 mg/mg
I	0 538	–	0 493	3092	575 ± 7	1 091 ± 0 010
II	0 526	0 490	0 481	3020	574 ± 13	1 095 ± 0 021

distributed (see Table I) Since the calculated intake of ^{45}Ca depends on the ratio of the dietary contents of ^{45}Ca and chromic oxide it is of interest to note that analyses taken at the beginning and the end of the experiment gave practically identical values for this ratio (compare the ratios $^{45}Ca/Ca$ and Ca/Cr_2O_3 Table I)

The remainder of the diet to which no isotope was added was fed to the rats between metabolic periods The rats regularly received 150 I U vitamin A acetate in oil every 2 days

Parathyroidectomy, parathyroid extract and blood samples

The parathyroids were removed by hot wire cautery as described by Munson (195[illegible]) and Hirsch Gauthier and Munson (1963) The parathyroid extract was assayed by Eli Lilly Co to contain 375 units per ml The rats received doses by subcutaneous injection varying between 375 and 1 100 units once daily or every 3 days during the third stage of the experiment Blood samples of 1–1 5 ml were taken from the tail while the rats were lightly anesthetized with ether

Analytical procedures

All feces and urine from a metabolic period as well as diet samples were digested first with nitric acid and then with perchloric acid (Gran 1960 a) Since this procedure leads to oxidation of chromic oxide (Cr^{3+}) to chromic anhydride (Cr^{6+}) extreme care was exercised to prevent distillation and loss of chromic anhydride during the last stage of digestion All analyses were carried out at least in duplicate except those of serum calcium for which only one serum aliquot was available

1 *Chromic oxide*

After alkalinization the concentration of sodium chromate was determined at a wave length of 372 millimicrons in a Beckman DU spectrophotometer When chromic oxide was added to mixtures of wheat flour and oil in amounts comparable to those of the diet recovery of this material ranged from 98 3 to 99 0 per cent in 3 tests In 6 other tests recovery of 50 or 150 mg chromic oxide from mixtures with calcium carbonate and potassium dihydrogen phosphate (in ratios similar to those of the diet) was in the range 99 0–99 5 per cent

2 *Phosphorus*

The phosphorus content of the diet was determined by the method of Fiske and SubbaRow (1925) modified so as to assure complete hydrolysis of any pyrophosphate formed during digestion

3 *Calcium in feces diet and serum*

Untreated samples of serum and solutions of ashed feces and diet were analyzed for total calcium according to the original method of Munson *et al* (1955) The procedure involves titration with the disodium salt of EDTA with ammonium purpurate as indicator and is carried out in a Coleman Junior spectrophotometer

The combined procedures for ashing and determining calcium in samples of feces and diet were tested with a mixture of calcium carbonate potassium dihydrogen phosphate and calcium and phosphate free salts in concentrations comparable to those of the diet The recovery of calcium from 4 such samples ranged from 99 6 to 102 0 per cent

4 *Calcium in urine*

For reasons not clearly understood, the EDTA method proved unreliable when used for determining calcium in solutions of ashed urine Modifications designed to overcome magnesium interference as advocated by Kenny and Cohn (1958) were tried but found un successful However the procedure of Munson *et al* (1955) was found satisfactory when it was preceded by oxalate precipitation of calcium in the urine samples The precipitate was washed twice with dilute ammonia and dissolved in dilute sulfuric acid prior to titration with EDTA Recovery of calcium added to 4 solutions containing phos phorus (as dihydrogen phosphate) and magnesium in concentrations several times that of calcium (P/Ca > 10/1 Mg/Ca > 4/1) was in the range 99 3 to 101 2 per cent Calcium was also added to 12 solutions of ashed urine thereby increasing the calcium content by 70 per cent 170 per cent or 470 per cent The average recovery of the amounts added was 101 0 per cent (range 99 0–103 4 per cent)

5 *Radio-calcium*

A preparation of 8 g calcium carbonate containing 1 microcurie as ^{45}Ca was obtained from the Joint Establishment of Nuclear Energy Research at Kjeller Norway By adding hydrochloric acid and water a working solution of ^{45}calcium chloride was obtained which con tained approximately 0 06 mg Ca per ml

The handling of isotope containing solutions and materials and the determination of ^{45}Ca were carried out according to the re commendations of Comar (1955) and Gran (1960 a) In general the counting was preceded by the complete oxalate precipitation of calcium in an aliquot of a digested sample to which had been added 4 mg of carrier calcium The precipitate was washed once with distilled water transferred quantitatively to aluminium dishes and allowed to dry slowly The radioactivity was measured by a thin end window GM tube with a mica thickness of 1 59 mg/cm² The rates were corrected for background activity (approximately 13 counts per min) coincidence losses decay and self absorption Most samples had a thickness of 3–5 mg/cm² which gave from 10 to 16 % loss by self absorption

Ten series of 2 000–4 000 counts each were recorded for each of duplicate samples of urine feces and diet The standard deviations (counting errors) in 7 series of 17 recordings of 4 000 counts ranged from 1 3 to 2 2 per cent the rates varying from 240 to 2 500 counts

per minute With the exception of a few samples, mostly urine, all counting rates were within this range

The procedures for digestion, oxalate precipitation and plating were tested by determining the recovery of 4 ml of the working isotope solution added to a 5 g portion of diet in a digestion flask Compared to the results obtained by counting directly plated samples the recovery was 101 6 per cent (average of 5 duplicate samples) The precipitate thickness was greater than that for any of the samples from the actual experiment

Calculation of coefficient of true absorption (X)

Since Equation 1 includes the term Ca_s (digestive juice calcium) the coefficient of true absorption must be expressed by a different equation containing quantities which can be measured directly The following derivation is taken from Gran (1960 a)

The feces calcium may be expressed as follows

$$Ca_f = Ca_i - Ca_a + Ca_s - Ca_{sa}$$

or (Eq 2)

$$Ca_f = Ca_i - Ca_a + Ca_e$$

where Ca_a and Ca_{sa} are the amounts of ingested and secreted calcium which are absorbed and Ca_e is the endogenous calcium in feces

X may be written

$$X = \frac{Ca_a}{Ca_i}$$

or

$$X = \frac{Ca_{sa}}{Ca_s}$$

which allow substitutions for Ca_a and Ca_{sa} in Eq 2 which then may be written

$$Ca_f = Ca_i + Ca_s - X(Ca_i + Ca_s)$$

Rearrangement of this equation results in

$$Ca_s = \frac{Ca_f}{1-X} - Ca_i \qquad \text{(Eq 3)}$$

The total radioactivity in the feces is (see Eq 2)

$$^{45}Ca_f = {}^{45}Ca_i - {}^{45}Ca_a + {}^{45}Ca_e \qquad \text{(Eq 4)}$$

Assuming that the specific activity for Ca in the digestive juices is the same as that in plasma

$$^{45}Ca_e = Ca_e \; S\,A_{pl} \qquad \text{(Eq 5)}$$

Since

$$Ca_e = Ca_s - Ca_s \; X$$

and according to Eq 3 Eq 5 may be written

$$^{4}Ca_e = (1 - X) \;\; S\,A_{pl}\left(\frac{Ca_f}{1-X} - Ca_i\right)$$

This expression and the following term

$$^{4}Ca_a = X \;\; ^{4}Ca_i$$

are then substituted in Eq 4 which then becomes

$$^{4}Ca_f = {}^{45}Ca_i - X \;\; ^{4}Ca_i + S\,A_{pl} \;\; (1 - X)\left(\frac{Ca_f}{1-X} - Ca_i\right)$$

from which X may be solved

$$X = \frac{^{4}Ca_i - {}^{4}Ca_f + (Ca_f - Ca_i) \;\; S\,A_{pl}}{^{45}Ca_i - Ca_i \;\; S\,A_{pl}} \qquad \text{(Eq 6)}$$

It appears from Equation 6 that when the specific activity of the plasma increases the precision of the method decreases mainly because $Ca_i \; S\,A_{pl}$ in the denominator reaches high values In con trast to the experiments of Gran (1960 a) the specific activity of the plasma was generally high and declining rapidly during one stage in the present experiment (Fig 2) A correction for the urine feces time lag was therefore used

Statistical tests

1 *Modified t test*

In order to test the significance of the observed difference in coefficient of true absorption between stage 1 and 2 the following t test was used

$$t = \frac{(\bar{X}_1 \;\; - \bar{X} \;\;)}{S} \sqrt{\frac{2n_1 \;\; n_2}{n_1 + n_2}} \qquad \text{(Eq 1)}$$

where $\bar{X}_1$ is the average of all observations at stage 1 (periods 2 and 3 combined) and $\bar{X}_2$ is the average of all observations at stage 2 (periods 6 and 7 combined). Subscript i refers to the numbering of the stages (1 or 2), subscript j refers to the numbering of the rats at each stage, n_i (i.e. n_1 and n_2) is the number of rats in the first and second stage respectively, and subscripts 1 and 2 refer to the first and second observation for each rat at each stage. Finally, S is given by

$$S = \frac{1}{2}(n_1+n_2)\sum_{i=1}^{2}\sum_{j=1}^{n_i}(X_{ij1}-X_{ij2}) \qquad \text{(Eq. 2)}$$

The number of degrees of freedom is $(n_1 + n_2) = 15$.

2. *Analyses of correlation*

The total correlation coefficients for digestive juice calcium–serum calcium (r_{XY}), digestive juice calcium–food consumption (r_{XZ}) and serum calcium–food consumption (r_{YZ}) were calculated according to the equation

$$r = \frac{\sum_{i=1}^{n}(X_i-\bar{X})(Y_i-\bar{Y})}{\sqrt{\sum_{i=1}^{n}(X_i-\bar{X})^2 \sum_{i=1}^{n}(Y_i-\bar{Y})^2}} \qquad \text{(Eq. 3)}$$

where n is the total number of observations (25) and $\bar{X}$ is the mean of all observations of X. On the basis of the total correlation coefficients the partial correlation coefficients were calculated as follows:

$$r_{XY.Z} = \frac{r_{XY} - r_{XZ}\,r_{YZ}}{\sqrt{(1-r_{XZ}^2)(1-r_{YZ}^2)}} \qquad \text{(Eq. 4)}$$

After the partial coefficient for digestive juice calcium–serum calcium had been calculated, the subscripts (Y and Z) for serum calcium and food consumption were switched to allow calculation of the partial coefficient for digestive juice calcium–food consumption. The number of degrees of freedom under testing is $(n - 3) = 22$.

The statistical tests have been carried out according to the recommendations by Hald (1952).

Experimental plan

Figure 2 which shows the plasma specific activity for calcium, shows the sequence of the stages of the experiment At the end of the 7 month pre treatment period 10 rats were allowed to become familiar with the balance cages, the balance routine as described by Gran (1960 a) and the purified diet The isotope containing diet was subsequently introduced and feces and urine collections for period 1 were started 2 days later Each collection period (except for period 12) lasted 3 days At the end of periods 3 and 4 the rats were given the isotope free diet for 15 and 16 days respectively so as to prevent excess accumulation of isotope by the skeleton while the collected urine and feces were analyzed Metabolic periods 1–4 constitute the control stage The isotope diet was then re introduced 2 days before the rats were subjected to parathyroidectomy, and they were kept on this diet for the remainder of the experiment Period 5 represents feces and urine collected between 3 and 6 days after the operation Period 8, for which only urine samples were available marks the end of the second stage of the experiment On the following day the rats were given an initial injection of 1 100 units parathyroid extract The average maintenance dose thereafter was 185 units per day the frequency of injections ranging from daily to every 3 days The variation in treatment stems from the attempt to achieve maximal hypercalcemia by treating each rat according to the results of frequently taken blood samples From day 75 on the remaining 8 rats were given doses of 600 units per day, with the result that only 5 rats survived the end of the experiment

Results

Serum calcium

The rats were bled twice before and twice after parathyroidectomy and all except one rat were bled at least twice during the stage of extract treatment (see Figure 1) From a slightly subnormal level of approximately 9 mg/100 ml the serum calcium concentration decreased to 5 1 mg/100 ml 6 and 12 days after parathyroidectomy The result of the extract injections was a clear return of the serum calcium level to the normal range except at the time of the last

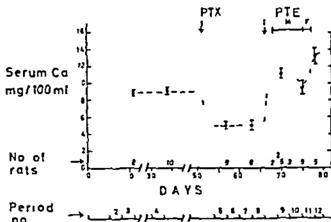


Fig 1 Effect of parathyroidectomy (PTX) and injection of parathyroid extract (PTE) on the serum calcium of vitamin D-deficient rats
The dots and vertical lines represent the mean and twice the standard error respectively The doses of parathyroid extract were 1 100 units as initial dose (I) 300 units every 2 days as maintenance dose (M) and 600 units per day as final dose (F)

blood sample when in response to the increase in dose the average level was found well within the hypercalcemic range The primary objective of the experiment the change from a hypoparathyroid state to a hyperparathyroid state was presumably achieved It was also of great interest to note that in spite of the vitamin D deficiency the rats responded to removal of the parathyroids by a substantial fall in serum calcium The subsequent rise in serum calcium after extract injections indicates that the commercial extract as well was active in the presence of only minute amounts of vitamin D This conclusion has been amply supported by subsequent experiments carried out in this laboratory and presented in detail elsewhere (Toverud 1964)

Specific activity of calcium in plasma

Figure 2 shows the specific activities of calcium in urine which were assumed equal to the specific activities of calcium in plasma There was a marked increase until the 8th–11th day when the curve began to level off Gran (1960 a) observed a steady and much slower rise when he used a diet containing the same specific activity one tenth as much total calcium and 0 5 per cent ammonium oxalate The rapid rise in Figure 2 was probably due to the high intake and absorption of the isotope

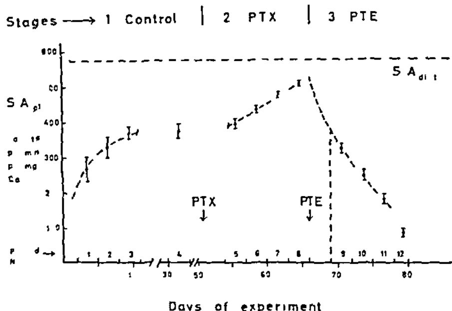


Fig 2 Specific activity of ⁴⁵Ca in plasma before and after parathyroidectomy (PTX) and injection of parathyroid extract (PTE) in vitamin D deficient rats
S A pl = Specific activity of plasma
S A diet = Specific activity of diet
The dots and lines represent the mean and twice the standard error
Note the discontinuity in the curve due to feeding of isotope free diet for 15 and 16 days at the end of days 11 and 33 respectively

Since the ^{45}Ca ingestion was discontinued from the 12th–26th day and the 34th–49th day the curve has been interrupted However it is remarkable that the specific activity in periods 4 and 5 is practically on a level with the specific activity in period 3

The effect of parathyroidectomy was a marked and steady rise, which may be explained by a reduced bone resorption with reduced dilution as a consequence Following injections of parathyroid extract there was a precipitous fall in plasma specific activity This was probably due to increased resorption of unlabeled or low activity bone mineral

The accentuated fall in the curve between periods 11 and 12 corresponds well with the rise in serum calcium shown in Figure 1

The rise and fall in plasma specific activity associated with parathyroidectomy and parathyroid extract injections respectively, would seem to be consistent with the idea that parathyroid hormone primarily stimulates resorption of the relatively stable fraction of bone mineral (Munson Hirsch and Tashjian 1963)

The variations in plasma specific activity from period to period made it imperative to correct the activity for the time lag between urine and feces (length of time required for the passage of feces through

the intestines) A separate test with the use of carmine as indicator, showed the lag to be approximately 1 5 days or one half of a metabolic period with wide individual variations In calculating the coefficient of true absorption, the average of the specific activity for the current and the preceding period for each rat was used The inaccuracy introduced into the results by this method of correction will be discussed below Since there were no figures available for the 3 days preceding period 9 corrected figures for specific activity were obtained as follows By extending the activity curve (Figure 2) backwards 1 5 days an average value of 380 counts per minute per mg calcium was obtained The uncorrected activity value for each rat was then multiplied by 380/328 the figure 328 being the mean uncorrected activity for period 9

Absorption and excretion of total calcium

Figure 3 shows that the average calcium intake varied around 50 mg in all three stages

The figures for urinary calcium excretion were also corrected for the urine feces lag of 1 5 days so that for each period the reported figure represents the average of the current and the preceding periods (except periods 4 5 and 7 for which results from the immediately

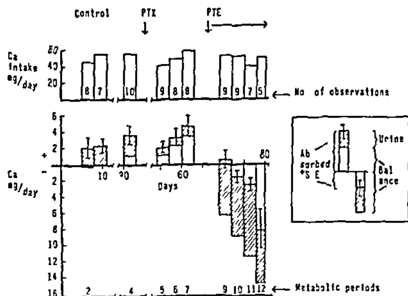


Fig 3 Calcium balance before and after parathyroidectomy (PTX) and after injections of parathyroid extract (PTE) in vitamin D-deficient rats

The width of the bars indicates the length of the metabolic period and the height represents the mean for the indicated number of rats The vertical lines represent twice the standard error of net calcium absorption

preceding 3 days were not available) The difference between the corrected and the uncorrected figures for each period did not exceed 0 16 mg in periods 2 3, 6 and 7 and 2 3 mg at stage 3 The urinary calcium represented by the cross hatched columns, appeared to decrease after parathyroidectomy Following parathyroid extract injections it increased to about 3 times that of the control periods These changes which paralleled the general serum calcium levels represented the chronic effects of parathyroidectomy and injections of high doses of parathyroid extract respectively Talmage and Kraintz (1954) found a tendency towards similar effects after 25–27 hours, while the acute effects in their study consisted of the reverse trends namely an increase after parathyroidectomy and a decrease after parathyroid extract injection The present results are thus consistent with the concept that parathyroid hormone enhances the renal tubular reabsorption of calcium and that the rate of calcium excretion is the net result of an increased amount of calcium filtered and increased rate of reabsorption (Bernstein Kleeman and Maxwell 1963)

The mean net absorption of total calcium represented in Figure 3 by columns fitted with lines for the standard error, varied between approximately + 2 and + 5 mg in stages 1 and 2 There was a tendency towards an increase in calcium balance during stage 2 mainly because of the decrease in urinary calcium excretion During treatment with parathyroid extract the rate of net absorption decreased and the rats exhibited a net loss of calcium with the feces Because of the concurrent increase in urinary calcium excretion a high negative balance was observed

Coefficient of true absorption

Table II contains the values for the coefficient of true absorption for the different periods During the control stage the mean values were 0 274 and 0 298 with wide variations between rats and between periods for each rat After parathyroidectomy the mean values were 0 193 and 0 203 which represent a decrease of approximately 28 per cent compared with the control stage This decrease was significant ($P < 0\ 01$) as judged from a t test based on both periods for each stage (Eq 1 Statistical tests)

If values for specific activity of calcium in plasma were left u corrected for the 1 5 day time lag and then used for calculation of t coefficient of absorption the decrease from stage 1 to stage 2 wo have represented approximately 25 per cent The time lag correc thus had little influence on the relative levels of true absorption l and after parathyroidectomy

Table II Effect of parathyroid hormone on true absorption of calcium and on digestive juice calcium in vitamin D-deficient rats

Mean and standard error for each period Table IV contains examples of the basic measurements from which the coefficients of true absorption were calculated

Stage	Period	No of rats	Coefficient of true absorption[1]	Digestive juice Ca mg/day	Digestive juice Ca corr[2]
1 Control	2	8	0 254 ± 0 010	12 0 ± 1 7	15 6
	3	7	0 298 ± 0 014	20 2 ± 1 4	19 9
2 PTX[3]					
days after					
6–9	6	8	0 193 ± 0 026	7 6 ± 1 7	8 3
9–12	7	8	0 203 ± 0 029	10 7 ± 3 2	9 8
3 PTX+PTE[4]					
days after					
1st injection					
3–6	9	9	0 246 ± 0 026	17 2 ± 2 5	17 3
6–9	10	9	0 223 ± 0 015	16 5 ± 2 3	16 9
9–12	11	7	0 214 ± 0 018	13 5 ± 2 0	18 4
12–14	12	5	0 135 ± 0 015	17 1 ± 2 5	18 5

[1] Calculated from Eq 6
[2] Expressed in mg/day/10g diet intake
[3] PTX=parathyroidectomy
[4] PTE=parathyroid extract

The present results therefore indicate that removal of the parathyroids of vitamin D deficient rats significantly reduced the true absorption of calcium Such a reduction in absorption has also been obtained with other techniques *in vivo* (Cramer 1963) and *in vitro* (Rasmussen 1959 and Schachter 1963) in vitamin D supplemented rats In the absence of vitamin D only a small and not significant reduction in transport of calcium in everted gut sacs was caused by prior parathyroidectomy (Dowdle Schachter and Schenker 1960) However Kodicek (1964) in studying absorption of ^{45}Ca from intestinal loops *in vivo* also found evidence of a significant effect of removal of the parathyroids in vitamin D deficient rats

After some parathyroid extract injections had been given the coefficient of true absorption appeared to increase (period 9 Table II) However the values for the subsequent periods were as low or lower than those observed at stage 2 In view of the considerable variability during this and the control stage, it must be concluded that parathyroid extract had no effect on true absorption

The reason for the failure to demonstrate an increased true absorption may be that the rapid decline of the plasma specific activity makes it necessary to use a time lag correction of more than 1 5 days If 3 days is used in period 10, the coefficient of true absorption becomes 0 263 instead of 0 223 Although a time lag of 3 days is highly im probable, this calculation illustrates the relative reliability of the values for true absorption at this stage

It is noteworthy that others who have demonstrated decreased absorption following parathyroidectomy have also failed to restore absorption with parathyroid extract (Rasmussen 1959 and Schachter 1963)

Digestive juice calcium

It is well established that the digestive juice calcium varies with food intake (Gran 1960 a) and the figures in the last column (Table II) represent values per 10 g food intake

The reduction of digestive juice calcium following parathyroid ectomy is striking Since digestive juice calcium is dependent upon the plasma calcium level (Gran 1960 a) the result is an expected one

Following parathyroid extract injections, digestive juice calcium increased to the range for the control periods This increase paralleled the general rise in serum calcium A direct comparison for each period between the digestive juice calcium values in Table II and the serum calcium in Figure 1 cannot be made since serum calcium values were not available from all rats

The range of mean values of digestive juice calcium in this experiment is fairly similar to that reported by Gran (1960 a) for vitamin D deficient rats (5–12 mg per day)

Table III Analysis of correlation between digestive juice calcium food consumption and serum calcium in vitamin D-deficient rats

Serum calcium values were available only during periods 3 7 10 11 and 12 thus providing 25 observations for the analysis The partial correlation coefficient (partial r) is the total correlation coefficient (total r) corrected for the effect of the third quantity according to Eq 4 (Statistical tests)

Quantities	Total r	Partial r
Ca_g[1]—Serum Ca	0 501	0 628 P $\approx$ 0 001
Ca_g—Food	0 403	0 569 P $<$ 0 01
Serum Ca—Food	− 0 165	

[1] Digestive juice calcium

Correlation between digestive juice calcium, food consumption and serum calcium

The data from period 0 were excluded from the analysis of correlation because of the special manner in which the plasma specific activity for this period was corrected for the lag period Of the remaining periods only 3, 7, 10 11 and 12 coincided with serum calcium de terminations Table III contains the correlation coefficients for the 3 quantities—digestive juice calcium serum calcium and food con sumption By expressing the relationship by the partial correlation coefficient the effect of the third quantity was eliminated Both partial correlation coefficients are positive and significantly different from zero Consequently the results of this analysis support the con clusion by Gran (1960 a) that the amount of calcium secreted with the digestive juices varies both with the serum calcium level and the daily food consumption

Discussion

Method for calculating true absorption

The use of the t test for the effect of parathyroidectomy on the coefficient of true absorption (λ) presupposes a normal frequency distribution of λ at each treatment stage and that the range of λ has definite minimum and maximum limits Since both conditions presumably are satisfied in this case application of the test may be considered valid

The variation of λ between periods and between rats expressed as the variance of λ (S^2) (see statistical tests) is the net result not only of the biological variation but also of errors in all components of the equation from which λ is calculated (Eq 6) The result of the t test and the conclusions drawn from it will thus be valid regardless of the degree of influence on λ of any one error However in the event of future use of this method an analysis of these errors seems indicated

As noted in the introduction the higher the plasma specific activity the less the precision of the method During stage 2 when plasma specific activity approached diet specific activity the precision may have been particularly low In order to compare the effects on the

Table IV Influence of analytical errors on the coefficient of true absorption (λ)
The mean value of λ for each of 3 periods has been calculated from the mean analytical values as shown in the top section of the Table[1] (See Equation 6) Each value has been reduced one at a time by 1 per cent and the percentage difference between the resulting value of λ and the actual value of λ has been recorded in the bottom section of the Table

Period No	S.A.$_{pl}$ counts per min per mg	Food intake g	Ca_f mg	$^{45}Ca_f$ counts per min	λ
2	300 7	8 299	42 89	22 022	0 254
7	455 9	10 945	54 23	30 305	0 203
10	288 5	9 753	53 90	27 229	0 223
2	0 8	2 8	3 9	7 1	
7	2 0	3 4	16 7	21 2	
10	1 3	3 6	4 9	8 1	

[1] The diet contained 0 5375 per cent calcium and 3 091 counts per min per g ^{45}Ca

absorption coefficient (λ) of different analytical errors the average values from the 4 independent analyses (Ca $^{45}Ca_f$ S A $_{pl}$ and diet intake) for periods 2, 7 and 10 were used Table IV shows the per cent change in λ resulting from a 1 per cent change in each of the 4 measurements The greatest effects on λ are found in period 7 as expected while the results for periods 2 and 10 are quite similar Regardless of the period λ appears to be most sensitive to changes in $^{45}Ca_f$ and least sensitive to changes in the specific activity of plasma The latter finding is of particular interest since the actual value for S A $_{pl}$ was based partly on a direct measurement and partly on the assumption that the time lag between urine and feces was always 1 5 days One can therefore conclude that errors in estimation of the lag have apparently played a relatively small role

The high degree of dependence of λ on errors in $^{45}Ca_f$ shows how necessary it was to determine this amount with a high degree of accuracy each of the duplicate samples of every batch of feces was counted 10 times until a total of 40 000 counts was registered The large error in λ resulting from the 1 per cent reduction in the value for Ca_f in period 7 was probably due to the combination of the high value for S A $_{pl}$ and the relatively great difference between Ca_i and Ca_f

Theoretically the following combination of values should give the most accurate estimate of λ low Ca_i low S A $_{pl}$ low (Ca_i—Ca_f) and

high $^{45}Ca_f$ However the combination of a low rate of net absorption of total calcium a high rate of isotope absorption and low levels of plasma specific activity will obviously be impossible to achieve Furthermore a reduction in calcium intake may lead to loss of precision in determining feces calcium

One of the assumptions that forms the basis of this method for calculating true absorption is the complete mixing of ingested calcium with secreted calcium Mixing is probably complete in the stomach and duodenum If most of the calcium is absorbed in the upper part of the small intestine (Lindquist 1952) an error may occur when calcium secreted in the lower part of the small intestine is mixed with non absorbed and precipitated calcium On the other hand should most of the calcium be absorbed in the lower part of the small intestine as contended by Marcus and Lengemann (1962) the error would be negligible

In the experiments presented here the uncertainties with regard to precision are very largely due to the relatively high specific activity of the plasma In consequence any error due to addition of intestinal juice calcium would be smaller than when specific activity of the plasma is very low

Ion exchange between luminal contents and the intestinal wall has been considered to be a serious error of this method However Gran (1960 a) has shown that a three fold increase in specific activity of plasma did not significantly affect the calculated value for digestive juice calcium Isotope uptake by the intestinal wall by ion exchange should decrease with increasing plasma specific activity and if such isotope uptake had been appreciable this trend would have affected the values for digestive juice calcium With the relatively high plasma specific activity in the present experiment any error due to ion exchange would consequently be quite small

Net versus true absorption of calcium

Only in later years has the study of the true absorption of calcium in balance experiments become practicable In earlier years the term absorption was frequently used synonymously with true absorption Many valuable data resulted but erroneous conclusions have also been drawn

A discussion of the problem of net versus true absorption using varying values for calcium in the food in the digestive juices and for the true absorption may be useful Figure 4 illustrates the variation

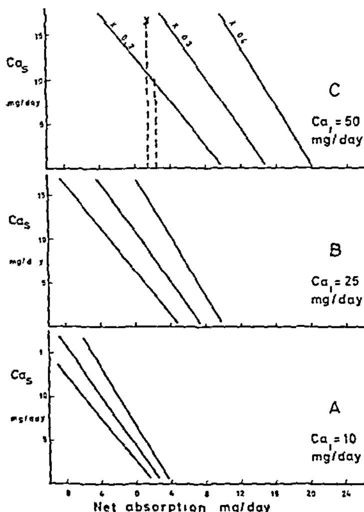


Fig 4 Variation in net absorption as a function of digestive juice calcium and of the coefficient of true absorption
Cai = calcium intake in mg/day
Cas = digestive juice calcium in mg/day
λ = coefficient of true absorption

in net absorption as a function of digestive juice calcium and of the coefficient of true absorption It appears that for any given coefficient of true absorption net absorption increases when digestive juice calcium decreases When the calcium intake is 50 mg daily and the digestive juice calcium is constant (15 mg/day) a doubling of the coefficient of true absorption leads to increased net absorption of 13 mg calcium whereas at the 10 mg dietary level the net absorption is only increased by 5 mg

The results from the present data may also be illustrated by Figure 4 The mean calcium intake was approximately 50 mg at stage 1 and 2

Parathyroidectomy was followed by a reduction in the coefficient of true absorption from 0 27 to 0 20 and in digestive juice calcium from 17 to 9 mg per day The resulting change in net absorption is very small, as was shown in Figure 3 Theoretically increased para thyroid activity leading to an increase in the coefficient of true absorption as well as in serum calcium could result in increased digestive juice calcium and thus little change in net absorption However this may not hold true if bone and intestine have different degrees of responsiveness to the hormone in different situations

Figure 5 illustrates variation in true absorption as a function of digestive juice calcium at given values of observed net absorption

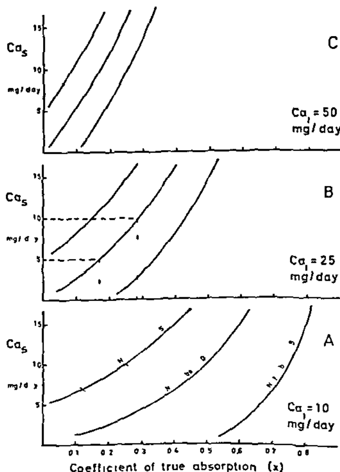


Fig 5 Variation in true absorption as a function of digestive juice calcium at given values of observed net absorption

Cai = calcium intake in mg/day
Cas = digestive juice calcium in mg/day
Net abs = net absorption in mg/day

Digestive juice calcium

In Cramer's (1963) experiments the rate of appearance of calcium in the lumen of Thiry Vella loops perfused with distilled water was low but constant over a wide range of plasma calcium (6–15 mg/100 ml). The author interpreted this observation as support of the view by Nicolaysen Eeg Larsen and Malm (1953) that the gut does not play any active excretory role with regard to calcium, and he postulated that the calcium appearing in the lumen may at least partly represent secretion from gut wall glands. Even though this secretion of calcium containing fluid from intestinal glands is occurring at a low, resting rate, one would have expected the secretion to reflect the concentration of calcium in plasma, from which it is derived. However, since it is possible that both the nature of the perfusing solution (distilled water) (Tidball 1963) and the short period of observation have influenced the results, there is little merit in trying to reconcile this study with the present one with regard to a relationship between serum calcium and digestive juice calcium. Furthermore Cramer's intestinal loops were removed from the influence of other intestinal secretions (exocrine juices), which, in the rats of the present experiment, must have contributed most of the digestive juice calcium. Cramer's work shows clearly however that there is no simple diffusion of calcium through the intestinal wall from the serosal to the mucosal side and neither is there an active excretory mechanism for calcium. Both conclusions are consistent with the results of the present experiment and the concept, maintained in this laboratory of the passive nature of the secretion of calcium with the digestive juices.

The influence of the parathyroids on calcium absorption which has been demonstrated in the present experiment and in those of other investigators appears to be small compared to that of other hormones and vitamin D (Schachter 1963) but it may nonetheless represent a homeostatic mechanism of some importance. Certain physiological states such as lactation may be accompanied by a reduction in skeletal calcium and also serum calcium. While the action of parathyroid hormone on bone contributes to the rapid restoration of a reduced calcium level in the body fluids the action on the intestine may perhaps contribute to the restoration of bone calcium (Cramer 1963).

As already suggested by Rasmussen (1961) the intestine may be one of many organs which respond to the fluctuating levels of circulating parathyroid hormone by influencing the transfer of calcium across biological membranes. An integrated concept of parathyroid action at the organ level (Rasmussen 1961) includes a response in

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LUND 1964
CARL BLOMS BOKTRYCKERI A.-B.

To My Father

Contents

Chapter I

General Introduction

This investigation is concerned with spinal reflex actions to motoneurones of foot muscles and deals in particular with reflexes evoked from the plantar skin.

The dominant reflex movement in response to skin stimulation in the hindlimb of an acute spinal cat is flexion — causing a withdrawal of the limb from the stimulus (SHERRINGTON 1900, 1910). This flexion reflex has been regarded mainly as protective. Reflex extension of the leg may be evoked by stimuli in the contralateral limb or ipsilaterally from genitocrural and perineal afferents but usually not as early after spinal transection as the flexion. More elaborate reflexes may appear several days after the transection, such as the extensor thrust described by SHERRINGTON already in 1900. Even stepping movements resembling those of the natural step can be evoked in the chronic spinal preparation by exteroceptive as well as proprioceptive stimuli (PHILIPPSON 1905 and SHERRINGTON 1910).

Investigations on the spinal preparation have the great advantage that any reflex found is known with certainty to be spinal. There is, however, now increasing evidence regarding the importance of descending control from higher centres of the transmission in different reflex paths (see chapter VI). Some spinal reflexes may depend on a particular type of supraspinal control in order to function and may not appear even in the chronic spinal animal.

Reflexes elicited from the foot are of special interest because of the role they may play in locomotion or maintenance of posture. The extensor thrust has already been mentioned, the magnet reaction and other components of the positive supporting reaction are other examples of such reflexes (cf. MAGNUS 1926 and RADEMAKER 1931). Tactile placing is also readily evoked from the foot (cf. BARD 1933). It is difficult to decide to what extent these reflexes are spinal. Tactile placing is generally considered to be a cortical reflex but this has not been proved satisfactorily. Only the extensor thrust can be evoked in the spinal animal. It consists of a brisk extension of the limb at hip, knee and ankle on light pressure

Contents

Chapter II

Methods

Two types of experiments were performed: A. Investigations of spinal reflex actions in motor nuclei. *B*. Investigations of muscular activity during locomotion.

For the investigations of spinal reflex actions in motor nuclei the conventional electrophysiological techniques were used for recording discharge, conditioning of monosynaptic test reflexes and intracellular recording.

In the experiments concerning muscular activity during locomotion a technique was used for leading off and telemetering muscle action potentials which was developed by research engineer L. LIDE and the author some years ago for analogous experiments (ENGBERG and LUNDBERG 1962).

A. *The investigations of spinal reflexes* were performed on unanaesthetized cats except in a few cases. During the dissection a carefully adjusted ether anaesthesia was given from an anaesthetic machine specially designed for cats. At the end of the operation the animals were either decerebrated by intercollicular section or anaemically decorticated (cf. VOORHOEVE 1960) and the anaesthesia was discontinued two hours or more before the start of any recording. The animals were then in many cases paralysed with Flaxedil and artificially respired. A few animals (declared in the text) were continuously kept in a light Nembutal or chloralose anaesthesia.

Normal body temperature was maintained by direct warming through the stand in which the animal was mounted and by exposure of the animal to infrared light when required. The blood pressure was continuously measured via a cannula in the common carotid artery on one side and a mixture of Macrodex and Rheomacrodex solutions was given in small amounts intravenously when vascular failure threatened.

The experiments were performed on acute spinal cats (transection in the 12th thoracic segment). Several muscular and cutaneous branches of the sciatic nerve were cut peripherally and dissected free. All the nerves were mounted on bipolar electrodes and covered with warm mineral

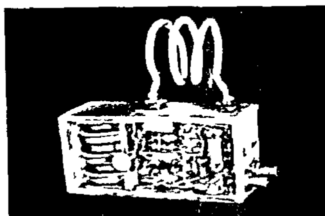


Fig. 1 A. *Transmitter for telemetering of electromyography from freely moving cats constructed by I. Eide.* The power supply consisting of three nickel-cadmium cells is seen to the left. Connections for amplifier input and battery charging are placed at the right end. Actual size.

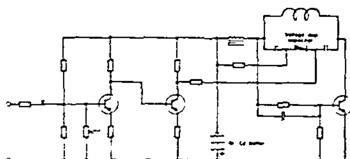


Fig. 1 B. *Circuit diagram of transmitter.* The oscillator (to the right) is frequency modulated by means of a voltage dependent capacitor fed from a two step preamplifier with an amplification of 30–100 times. The signal input has an impedance in the m[illegible] apparatus of 150–50 kΩ depending on the amplification. Total current drain [illegible] mA. Transmitting frequency 102 Mc. Together with a conventional FM receiver the test [illegible] high fidelity telemetering of electrophysiological data within a range of 50 m.

them were taken when necessary for subtraction from the intracellular record. The nerves were stimulated with a high repetition frequency in order to prevent reflex discharges in the motor nuclei.

B. The experiments concerning muscular activity in locomotion were performed in completely unrestricted animals. A fine enameled copper wire with a bare tip was inserted in the muscle during a short Fluothane narcosis before each recording, and the wire was soldered to the input

of a miniature amplifier transmitter fixed with tape to the back of the animal (A description of the transmitter is given in Fig 1) The wire was inserted inside a hypodermic needle A 1 mm hook bent at the end caused it to stay in the muscle when the needle was withdrawn The localization was controlled by palpation of the muscle and its tendon during stimulation through the electrode The reference electrode consisted of a copper ball ($\phi = 1$ cm) put into the rectum just inside the sphincters

The telemetered EMG signal was frequency modulated and received with automatic frequency control in order to obtain a steady recording The discriminated signal was recorded both directly and after integration in a simple rectifier capacitor system The animal was made to run along a pathway where it was photographed on a 16 mm moving film with accurate means of synchronization to the EMG recording

The analysis of the integrated EMG records required superimposing of several step cycles This was performed in a specially designed drawing apparatus in which records of different cycle length could be copied to a standard dimension

Chapter III

Anatomy and nomenclature

The experiments related in the following chapters mainly deal with the intrinsic foot musculature but also with some muscles with tendons descending from the leg that act in synergism with the short foot muscles at the toe joints. The generally accepted anatomical nomenclature of some of these muscles, flexors and extensors of the toes, does not agree with their physiological classification (see chapter V). In the present work the individual muscles will be defined according to the anatomical nomenclature (see REIGHARD and JENNINGS 1935, STROMSTEN 1947) but the general terms flexor and extensor will be used in their physiological meanings.

The intrinsic foot musculature at the plantar side is parted into two layers by the tendon of flexor digitorum longus (FDL). A schematic drawing of important tendons at the toes is seen in Fig. 2. The superficial layer consists of flexor digitorum brevis (FDB) and of six lumbrical muscles. FDB is a direct continuation of the plantaris tendon and gives off tendons inserting at the base of the second phalanx of each toe. These tendons are perforated by the 4 parts of the FDL tendon which insert at the terminal phalanges. Of the lumbricals three originate from the FDL tendon and insert at the three lateral FDB tendons; they have also been termed mm. accessorii or mm. interflexorii (ELLENBERGER and BAUM 1915, 1943). The other three lumbricals are more deeply situated and quite small, arising more distally where the FDL tendon divides, and they insert medially at the first phalanx of the three lateral toes.

Plantaris, FDB and the lumbricals are synergistic in moving the toes in a plantar direction, pulling on the first and second phalanges. FDL pulls in series with the lumbricals but has its strongest action via the perforating tendons to the end phalanges and is thus also of importance for the protrusion of claw.

The deeper musculature is dominated by the interossei which are not true interossei in the cat since they are situated entirely on the plantar side of the metatarsals. There is one interosseous muscle arising from each metatarsal bone, dividing distally into a lateral and a medial portion to

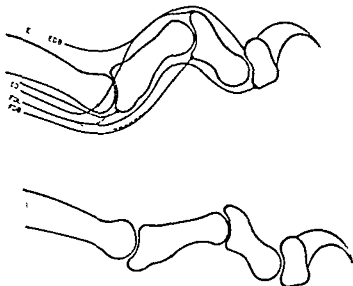


Fig 2 *Schematic drawing of a cat's toe* with part of the metatarsal ... of tendons and muscles (upper drawing) On stimulation of the ... angles of the different joints are changed to approximately the degree ... lower drawing

insert on both sides of the digit The muscles are not separ and plantar interossei as in the human foot It is possi distinguish between dorsal and ventral parts of the inter parts insert basally at the proximal phalanges pulling the direction whereas the ventral parts insert via tendons pa side of the metatarso phalangeal joints to join the com neurosis of the digit (i e joining the tendons of extensor and brevis) *These tendons move the second and third ph* direction and have hardly any effect on the position of th The lateral part of the interosseus of the fifth toe has dorsal aponeurosis It performs only a plantar flexion of and has therefore been termed flexor brevis digiti quinti and SPRAGUE 1944)

Superficial to the interossei are three smaller muscles adductor digiti quinti and adductor opponens digiti quin been described as a part of the first interosseus (REIGHA 1935) and inserts laterally on the basal phalanx of th other two insert medially on the basal phalanx and meta toe respectively A very small muscle quadratus planta proximally in this layer and pulls the FDL tendon lat in position when the latter muscle contracts

The innervation of the plantar muscles takes place via the medial and lateral plantar nerves both being branches of the tibial nerve. The lateral plantar nerve gives off a *deep main branch* which runs medially between the interossei and the adductors and gives off branches to all of them. FBV usually receives one or two separate branches that take off proximal to the interosseus innervation and the lateral parts of FDB may have several very small branches also from the lateral plantar nerve. The medial plantar nerve shortly after the bifurcation gives off a discrete nerve bundle with short branches to FDB and a long branch to the lumbricals.

The plantar cushion is mainly innervated from the medial plantar nerve by branches entering its centre via the dense connective tissue deep underneath the cushion.

The following abbreviations of anatomical names will be used:

ABSm = anterior biceps and semimembranosus
DP = deep peroneal nerve
EDB = extensor digitorum brevis
EDL = extensor digitorum longus
FBV = flexor brevis digiti quinti
FDB = flexor digitorum brevis
FDL = flexor digitorum longus and flexor hallucis longus
G S = gastrocnemius and soleus
IO = interosseus
IPM = intrinsic plantar muscles
L = lumbrical
Lat. pl. = lateral plantar nerve
Pl = plantaris
PS = superficial peroneal nerve
PBSt = posterior biceps and semitendinosus
Q = quadriceps or part of it
Sur. m., Sur. l. = medial and lateral sural nerve
TA = tibialis anticus
Tib = tibial nerve
Pad denotes the plantar cushion (the central pad of the foot sole)
Toe pad denotes a peripheral toe pad

Chapter II

Reflexes evoked from the plantar cushion

THE TOE EXTENSOR REFLEX

Reflex movement

In the acute spinal cat pressure on the plantar cushion evokes a r movement of the toes When the foot is freely suspended the resting tion of the toes is such that the basal phalanx is slightly dorsifl whereas the middle phalanx is plantar flexed During the applicatio pressure on the pad this position is changed at the two proxima joints The movement is largest in the basal phalanx which is be plantar direction but there is also a clear movement in a dorsal dire of the middle phalanx (see Fig 2) The latter movement need no active however as the plantar flexion of the basal phalanx cau passive increase of tension in the common dorsal tendons of the that might give dorsiflexion of the second phalanx The claws are protruded i e no significant movement is observed in the end ph (which is dorsiflexed at rest) No steady ab or adduction is seen i toes although very small phasic movements are seen in both of directions indicating some asynchronous activity in muscles perfor medio lateral movements in the proximal toe joint The reflex wi called the toe extensor reflex

Adequate stimulus for the reflex

The toe extensor reflex is often very easily evoked The natural sti needed were tested at the beginning of most of the experiments with von Frey hairs revealed that even as light a pressure as 1 g or middle of the pad can evoke the reflex in some animals Consider stronger pressure is required if the stimulation is performed with flat object There is however a great variation in threshold bet different animals which sometimes can be related to the thickness o pad skin The sensitivity is slightly higher in the distal parts of the than in the proximal

There is an increase in the response if the mechanical stimulus is increased by pressing the pad between two fingers or with anatomical forceps. No appreciable decrement in the response has been noted during continuous stimulation for 10—15 seconds. Pinching of a small part of the pad with forceps is very effective. There is no difference in the type of response depending on which part of the pad that is stimulated. Except for a small predominance of the tibial toe at threshold stimuli all 4 toes take equal part in the reflex. In many experiments it was possible to evoke the toe extensor reflex from skin areas proximal to the pad but stronger stimuli, sometimes clearly nociceptive in character, were needed. The effect diminishes as the stimulus is applied centrally towards the heel, above which the response cannot be elicited. The reflex can neither be evoked from the pads or hairy skin of the toes nor from the foot dorsum. Gentle manipulation of the toes or metatarsals in order to stimulate proprioceptors in the foot is ineffective. Proprioceptive inflow can not in fact play any important role when the reflex is evoked by pinching of the pad, as this leads to practically no dislocation of deeper structures in the rest of the foot. The general pressure of a flat object against the pad is less effective than the same pressure localized to a small area where it causes a local deformation of more superficial structures. Hence there is good evidence that receptors in the skin evoke the reflex, but no evidence has been obtained for participation of receptors in deep structures. The increase in response with increased squeezing or pinching of the pad indicates that nociceptors in the region may add to the afferent inflow of the reflex. In increasing the stimulus, however, the reflex response is near its maximum when the stimulus reaches a nociceptive state as characterized by the first signs of a general flexor reflex of the limb.

A moderate pinching of the central pad, only sufficient to evoke the toe extensor reflex, never evokes any movement in the rest of the limb. When the stimulation is further increased to stronger pinching, a general flexor reflex is elicited causing withdrawal of the limb. This occurs rather suddenly on a gradual increase of stimulation when the toe extensor reflex already has attained its maximum, as judged from the plantar flexion of the toes. A very different situation was found on performing the same experiment with one of the toe pads. A general flexor reflex was evoked with forceps at about the same threshold as for the central pad, but weaker stimuli had no effect whatever. The flexion was in this case accompanied by a slight withdrawal of the stimulated toe. This was a very small movement, however, which appeared as the contrary of that evoked from the central pad. The same response was evoked by pinching other parts of the toes.

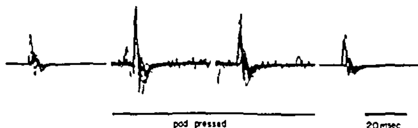


Fig. 3. Monosynaptic test reflexes of flexor digitorum brevis before, during and after the application of a moderate pressure on the plantar cushion. The reflexes were evoked by weak stimulation of the tibial nerve and recorded in the muscle. Each record consists of about 10 superimposed traces. The interval between each sweep was 0.5 sec and all traces are successive. Note the asynchronous discharge evoked in the muscle by pad stimulation.

Muscles participating in the reflex

In order to show which muscle groups of the limb that participate in the toe extensor reflex, many different motor nuclei were tested for their receptiveness of reflex actions from the planta. This was done in 20 experiments by conditioning monosynaptic reflexes with adequate stimuli to the central pad and its surroundings or with electrical shocks applied through needles in the pad. Furthermore, in the experiments with intracellular recording from motoneurones (chapter V) the synaptic effects of plantar stimuli were analysed.

a) *Plantar muscles.* Fig. 3 shows the effect of adequate pad stimulation on the monosynaptic test reflex of FDB. The pad is moderately squeezed between two fingers, as is indicated, and this causes a strong facilitation of the test reflex. The effect is almost unchanged as long as the pressure is maintained and there is also a continuous asynchronous discharge in the muscle.

It is possible to dissect away FDB and the tendon of FDL without interfering with the innervation of the pad and thus to get information of the movements in the underlying musculature upon pad stimulation. Reflex responses were seen in small muscles such as quadratus plantae, the adductors and the lumbricals and also in the interossei. The operation did not affect the toe movements in the reflex appreciably, which is readily explained by the large amount of muscle tissue in the interossei capable of plantar flexing the basal phalanges. A systematic exploration with small coaxial electrodes showed excitatory actions from the pad to all parts, dorsal or plantar, of the interosseus musculature. EMG record

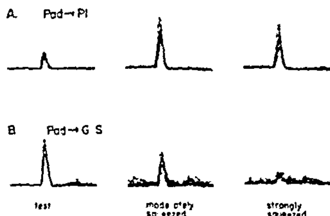


Fig. 4. *Monosynaptic test reflexes of plantaris (Pl) and gastrocnemius soleus (GS) recorded in the ventral root during moderate and strong squeezing of the plantar cushion.* The Pl test is strongly facilitated by the moderate stimulus. This facilitation is decreased by the noxious stimulus. The pure ankle extensor GS only receives inhibition from the pad. The discharge evoked in the ventral root by pad stimulation is more marked in the lower records since they were taken with higher amplification. Each record consists of several superimposed traces.

ings from the interossei clearly demonstrated the differences in reflex effects with stimulation of different skin areas of the foot. Pinching any of the toe pads strong enough to evoke a flexor reflex at the hip and knee does not activate the interossei. On the other hand gentle pinching of the central pad produces a massive discharge in these muscles without any reflex movements at the hip, knee or ankle. Stronger stimuli to the central pad or to the more proximal plantar skin gives an interosseus activation and at the same time a general flexor reflex.

b) *Crural muscles.* Motor nuclei of the following muscles were tested for their receptiveness to moderate pressure on the plantar cushion: GS, Pl, FDL, EDL, TA, peroneus tertius and peroneus brevis in the crus and PBSt, ABSm and Q in the thigh. Of these only FDL and Pl could be activated, and they both move the toes in a plantar direction. Discharges in the nerves to these muscles can be evoked by the same adequate stimuli that give effect to FDB. In many cats, however, the activation of FDL requires stronger pressure than needed for Pl and FDB. Facilitation of *a monosynaptic test reflex to Pl by moderate pad pressure is shown in* Fig. 4A.

Using electrical conditioning stimuli to the pad, monosynaptic testing was performed at different time intervals giving the curves of Fig. 5. The excitatory actions to FDL and Pl were never of the magnitude of

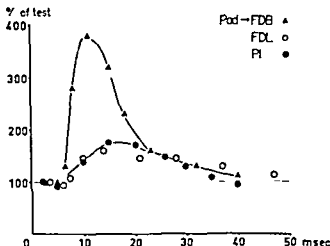


Fig. 3. *Excitability changes in the motor nuclei of the toe extensors flexor digitorum brevis (FDB), flexor digitorum longus (FDL) and plantaris (Pl) following an electrical stimulation of the plantar cushion.* The conditioned amplitudes of monosynaptic test reflexes (for FDB recorded in the muscle, for others in the ventral root) are expressed in per cent of the control amplitudes at each instance and plotted as a function of the time interval between conditioning and test *stimuli*.

those to FDB neither with adequate stimuli nor with electrical, but they have a similar time course.

In summary: Gentle pressure on the plantar cushion evokes reflex excitation in the motor nuclei of all muscles plantar flexing the toes. The receptors are situated in the skin, are sensitive to light pressure and are slowly adapting. Activation of nociceptors of the whole foot sole add to the afferent inflow of the reflex.

EFFECTS FROM THE PLANTAR CUSHION TO KNEE AND ANKLE MUSCLES

Throughout the whole series of experiments comparative tests were performed of the plantar effects to motor nuclei of hindlimb muscles without toe moving function. The monosynaptic reflexes of PBSt and G S have regularly been used for testing, and in a few cases those of ABSm and Q.

Adequate stimuli such as pressure or pinching applied to any part of the plantar surface, including the toes, never evoked excitatory actions in the motor nuclei of the extensors. Inhibition of the test reflex to G S was always elicited by a sufficiently strong squeezing of the pad. The threshold for this effect was variable but always higher than that for the toe extensor reflex. Fig. 4 compares the effects of moderate and

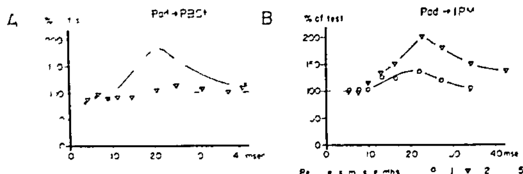


Fig. 6. *Excitability changes in the motor nuclei of posterior biceps semitendinosus (PBSt) and of the intrinsic plantar muscles (IPM) following electrical stimulation of the plantar cushion with different strengths.* Monosynaptic testing with recording in the ventral root. A conditioning strength twice as high as that needed to facilitate the IPM test has almost no effect on the PBSt test. Stronger stimuli facilitate both test reflexes. (Curves plotted as in figure 5)

strong pad pressure on the monosynaptic test reflexes of G S and Pl. A facilitation of the flexor PBSt parallels the inhibition of G S.

No great difference in sensitivity was noted between the central pad and other areas of the foot regarding the effect to PBSt or G S. In most experiments there was a clear difference, even when electrical pad stimuli were used, between the threshold for facilitation of IPM or Pl test reflexes on one hand and facilitation of PBSt or inhibition of G S tests on the other. This is illustrated in the curves of Figs. 6 and 7 from two different experiments. A conditioning stimulus of twice the strength necessary for facilitation of IPM (○ in Fig. 6 B) has hardly any effect on PBSt (A) but a stronger stimulus does evoke a facilitation to more than 150 % of the PBSt test. In Fig. 7 the same conditioning stimulation of the pad is used with three different tests showing only threshold effects to PBSt and G S but strong facilitation of Pl. Even in this case an increase in conditioning strength produces an appreciable facilitation of the PBSt reflex (*tested only at one interval*). Testing with reflexes to ABSm and Q gave results similar to those illustrated for G S.

It is concluded that muscles without toe moving function do not receive any reflex actions from the plantar cushion other than those expected from the flexor reflex afferents (FRA, cf. p. 26).

EFFECTS FROM THE PLANTAR CUSHION TO TOE DORSIFLEXORS

Two or three muscles of the limb, EDL, EDB and possibly peroneus tertius operate as antagonists at the toe joints to the muscles participating

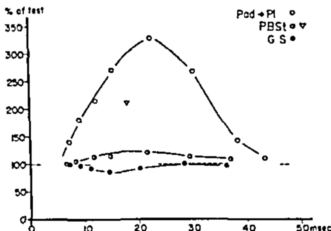


Fig 7 *Excitability changes in the motor nuclei of plantaris (Pl) posterior biceps semitendinosus (PBSt) and gastrocnemius soleus (GS) following electrical stimulation of the plantar cushion* Monosynaptic testing as in figure 6 The curves were all obtained with the same strengths of conditioning stimulus — evoking liminal actions in the PBSt and GS nuclei but a large excitation in the Pl motoneurones A stronger conditioning stimulus was applied at one time interval with the PBSt test (Marked ▽)

in the toe extensor reflex The reflex connections to FDL and EDB were studied in seven of the experiments using monosynaptic testing In most of the experiments their motor nuclei received excitation from the pad but no discharge was produced in the nerve to either of them by natural stimulation of moderate strength i e pinching with fingers or anatomical forceps enough to evoke a clear toe extensor reflex

In six of seven experiments a moderate facilitation of the EDB test appeared at a pressure slightly stronger than needed to evoke the toe extensor reflex and with still stronger stimulation there was a marked increase in this facilitation (Fig 8) About the same degree of facilitation was regularly evoked from the toes by the same stimuli and when tested in two experiments also from the rest of the planta and from the foot



Fig 8 *Monosynaptic test reflexes of extensor digitorum brevis (EDB) conditioned by adequate stimuli (as indicated) applied to the plantar cushion* The reflexes were evoked by weak stimulation of the deep peroneal nerve and recorded in the EDB nerve

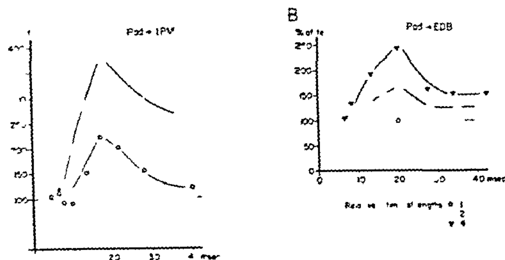
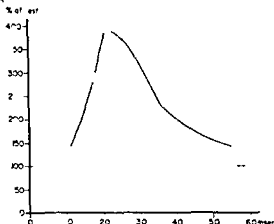


Fig. 9. Excitability changes in the motor nuclei of the intrinsic plantar muscles (IPM) and of extensor digitorum brevis (EDB) following single electrical shocks of different strength to the plantar cushion. Monosynaptic testing as in figure 6. The weakest stimulus used has no effect in the EDB nucleus but evokes marked excitation in the IPM nuclei. Stronger stimuli facilitate both test reflexes.

dorsum. Inhibitory actions from the pad to EDB were seen only in one experiment. In this cat (anaesthetized with Nembutal unlike the others) a gentle pressure of the pad had a weak inhibitory effect. Electrical stimulation of the pad was tried in all experiments and the strength graded from below to several times above the threshold for effect to EDB. Except in the anaesthetized cat referred to above, *inhibition of EDB could not* be evoked at any strength. The curves in Fig. 9 are all from the same experiment showing the effects of different conditioning strengths upon IPM and EDB tests respectively. The weakest stimulus used (marked ○) had no detectable effect on the EDB test reflex.

The EDB motoneurones receive more complex reflex actions from the foot. Excitatory effects were found to dominate from the pad in four of the seven experiments though mixed with some inhibitory in two of them. In the remaining experiments adequate pad stimuli evoked moderate inhibition of the test reflexes or no effect; extreme nociceptive stimuli were not tried. Inhibition to EDB was evoked in two of these cats from the lateral region of the entire foot including the lateral part of the central pad, whereas pinching of the skin at the inside of the foot gave excitation. In another experiment inhibition could be evoked only from the dorsolateral aspect of the foot while other skin regions gave excitation. Pinching of the pad produced facilitation of the EDB test in the latter cat and



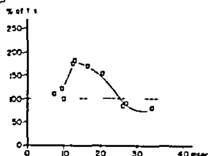


Fig. 10. *Excitability changes in the motor nucleus of extensor digitorum longus (EDL) following an electrical stimulation of the plantar cushion.* Testing with monosynaptic reflexes evoked and recorded in the EDL nerve. The curves are from two different experiments and illustrate the variations in response between different animals

this was most marked when the forceps were applied to the medial part of the pad. Electrical shocks to the pad in the same experiment produced the facilitation shown in Fig. 10 A. In two cats variation in stimulus strength altered the curve: strong stimuli gave a response similar to that in A, whereas weaker stimuli revealed an inhibitory component interrupting the early facilitation (B). It was not possible to find a correlation between the skin areas giving inhibitory effects to the EDL nucleus and the areas from which the toe extensor reflex is evoked.

TA was investigated in two cats. Adequate stimulation of either the central pad or the toe pads resulted in facilitation of the TA test. The effects were about equal to those of EDL in the same animals.

No evidence has thus been obtained for a reciprocal inhibition of antagonists in the toe extensor reflex. The facilitatory actions from the planta to these muscles presumably are part of the flexor reflex.

DISCUSSION

The experimental evidence presented in this chapter shows that the plantar skin predominantly at the proximal plantar cushion is the afferent source of at least two different spinal reflexes: the general flexor reflex and the toe extensor reflex. In the former, as is well known, muscles classified as flexors are activated in all parts of the limb, whereas the extensors are inhibited (SHERRINGTON 1910, ECCLES and LUNDBERG 1959a).

In the toe extensor reflex only muscles that plantar flex the toes participate. Two of these muscles, FDL and PI, have been classified as physiological extensors of the limb and it will be shown in the next chapter that the intrinsic plantar muscles also belong to this group. The flexor reflex needs stronger natural stimuli than the toe extensor reflex which is evoked by innocuous pressure. Gentle pressure on the plantar cushion raises the excitability in motoneurones of the short plantar muscles and of FDL and PI when no reflex action is seen to motoneurones of any other muscle group of the hindlimb. A possible function of this activation of all the plantar musculature would be to stabilize the foot during standing or during the stance phase of the step. Increased pressure on the pad towards the end of the stance phase would also, via this reflex mechanism, lead to the downward extension of the toes that is needed for completion of the extension phase of the step.

As stated on page 19, deformation of pressure receptors of slowly adapting type in the pad would be required for the effect. Recording of afferent impulses from such receptors in the most medial toe pad were made already in 1926 by ADRIAN and ZOTTERMAN. They distinguished two kinds of response in the digital nerve branch. A high frequency discharge occurred with light contact stimuli. This adapted very rapidly during 1/10 of a second and ceased within 1/2 a second. Continuous pressure of the pad could on the other hand produce a discharge that persisted for many seconds even though the frequency decreased rapidly during the first one or two seconds. MARUHASHI, MIZUGUCHI and TASAKI (1952) present similar records from "pressure fibres" (about 5μ thick), one originating in a toe pad, the other in the central pad. Pressure stimuli caused maintained discharges in these fibres, their frequency falling from a maximum of 30—60 to about 5 sec during continuous stimulation. They also describe rapidly adapting touch receptors in the pads and afferents that were activated only by noxious stimuli. No evidence has been obtained that the tactile afferents from the pad supply the reflex. It is concluded that the pressure afferents of these two investigations contribute to the toe extensor reflex.

The increase of reflex activation seen when the stimulus is increased from light to very strong pressure could correspond with the increase in adapted firing in the records of ADRIAN and ZOTTERMAN when they elevate the pressures to several hundred grammes. But the contribution of nociceptors can by no means be excluded. In particular it should be noted that the activation of toe extensors from the proximal planta often requires noxious stimuli. This could, however, also be a different reflex since HAGBARTH (1952) showed that stimulation of the skin area covering

a muscle generally evokes excitation to this muscle be it an extensor or flexor This may be part of a "purposive" organization of protective reflexes depending on what SHERRINGTON (1906) termed the "local sign" of the centripetal impulses

There is no evidence of a contribution in the toe extensor reflex from proprioceptors or any deep receptors in the foot This is in contrast to the reflex excitation of other extensors that can be elicited from the planta in the spinal animal — the extensor thrust (SHERRINGTON 1900 1903 1906) This reflex although it may be evoked by light stroking of the plantar skin is most readily obtained by a pressure between the plantar cushion and the toe pads which causes dorsiflexion of the toes and certainly involves an activation of proprioceptors Further no form of electrical stimulation is effective for the extensor thrust according to SHERRINGTON's *description There is also another important difference between* the two reflexes in that the latter "is never met with in the early time after transection" — it was usually not seen (in dogs) until three weeks or more after spinalization (SHERRINGTON 1900)

SUMMARY

The effect of adequate and electrical stimulation of the planta on motor nuclei of hindlimb muscles has been investigated with electromyography and with conditioning of monosynaptic reflexes

The plantar cushion constitutes a special region where light stimuli can evoke particular reflex actions to muscles that plantar flex the toes Gentle pressure on the pad evokes excitation in the motor nuclei of all the intrinsic plantar muscles and of FDL and PI Slowly adapting receptors situated in the skin are responsible for this effect Excitation in the same motor nuclei is also evoked by nociceptive stimulation of the plantar skin proximal to the pad

There is no evidence of a reciprocal inhibition in the motor nuclei of the antagonists EDB and EDL Stronger stimulation of the plantar skin evokes excitation in these motor nuclei

Hindlimb muscles without toe moving function receive no other reflex actions from the planta than those expected from the FRA in the acute spinal state (e g inhibition of extensors and facilitation of flexors)

Chapter V

Further analysis of synaptic actions to motor nuclei of foot muscles

INTRODUCTION

SHERRINGTON (1910) presented an analysis of reflex movements elicited from superficial as well as deeper receptor systems in the limb. These reflexes were all of a similar type, with flexion at hip, knee and ankle, and only slightly varying with different locations of afferent stimuli. In this flexion reflex of the limb, participation of a great number of muscles was demonstrated, including reciprocal inhibition of their antagonists. Based on this, and on their participation in reflex stepping, most muscles of the limb could be classified as flexors and extensors from the physiological point of view. Several electrophysiological investigations (LLOYD 1943; BROCK, ECCLES and RALL 1951; ECCLES and LUNDBERG 1959 a; PAINTAL 1961; and HOLMQUIST 1961) have contributed detailed information of the actions to different motor nuclei in the general flexor reflex, and of the afferent systems of importance for this reflex. These afferents, skin afferents, high threshold joint afferents and group II and III muscle afferents have been grouped together as the flexor reflex afferents (FRA). They have common polysynaptic actions not only to motoneurones but also to ascending spinal pathways (LAPORTE, LUNDBERG and OSCARSSON 1956; HOLMQUIST, LUNDBERG and OSCARSSON 1956, 1960; OSCARSSON 1957, 1958 and LUNDBERG and OSCARSSON 1960, 1961) and in reflexes to primary afferents (ECCLES, KOSTYUK and SCHMIDT 1962; CARPENTER, ENGBERG, FUNKENSTEIN and LUNDBERG 1963).

The reflex actions from the plantar cushion to certain muscles described in the last chapter differ in some respects from those that would be expected from the FRA according to the previous investigations. These investigations, however, did not include the pedal muscles to any great extent.

The motor nuclei of muscles participating in the toe extensor reflex and of their antagonists have therefore been subject to an investigation of synaptic effects from a variety of afferent inflow from the hindlimb

Apart from the analysis of effects from the plantar skin their receptiveness has been tested from other skin afferents from high threshold joint afferents and group II and III muscle afferents The aim has been to elucidate above all the participation of the different muscles in the general flexor reflex and to compare this to their reflex activation from the planta The long tendon muscles FDL FDL and PL which have been studied earlier during the investigations of the flexor reflex will only partly be dealt with here Most of the data have been obtained with intracellular recording from motoneurones but some results are derived from experiments with conditioning of monosynaptic reflexes

In addition the synaptic actions from group I muscle afferents on motoneurones of the plantar muscles were investigated in a few special experiments

Monosynaptic excitation in motoneurones of a certain muscle is evoked by the large muscle spindle afferents from this muscle (LLOYD 1943) but also to a certain extent by afferents arising in other muscles (ECCLES 1946 LLOYD 1946 a b LAPORTE and LLOYD 1952 JOB 1953 ECCLES *et al* 1957 a b and ECCLES and LUNDBERG 1958)

There is also reciprocal disynaptic inhibition in motoneurones of antagonist muscles (LLOYD 1946 a b ECCLES FATT and LANDGREN 1956 and ECCLES *et al* 1958) These reflex actions from Ia afferents may be of importance in muscle coordination and they have been studied extensively with intracellular recording in the motor nuclei of hindlimb muscles (ECCLES *et al* 1957 a b and ECCLES *et al* 1958) It has been demonstrated that excitatory Ia interconnections exist between FDB on one hand and PL and FDL on the other but the internal relations of the plantar muscles are unknown

The second group of large muscle afferents the Ib arises in Golgi tendon organs The conduction velocity of these fibres is only slightly lower than that of the Ia group and they have a somewhat higher stimulus threshold (BRADLEY *et al* 1953) The autogenetic inhibition caused by these afferents was described already in 1909 by SHERRINGTON and their synaptic actions have since been investigated by many In the early investigations (DENNY BROWN 1928 MATTHEWS 1933 MCCOUCH DEERING and STEWART 1950 GRANIT 1950 GRANIT and STRÖM 1951) the interest was focussed on the autogenetic inhibition but it has later been found that the autogenetic effects are not particularly prevalent (LAPORTE *et al* 1952 and ECCLES *et al* 1957 c) There is agreement that Ib afferents from extensors evoke widespread inhibition in extensor and excitation in flexor motoneu-

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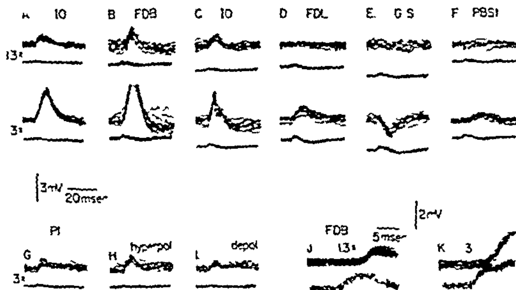


Fig. 11. *Intracellular recording from motoneurones of interossei (A, C), flexor digitorum brevis (B, J, K), flexor digitorum longus (D), gastrocnemius soleus (E), posterior biceps semitendinosus (F) and plantaris (G–I) showing synaptic actions by volleys in pad afferents.* Microelectrodes filled with potassium citrate. Ventral roots intact. (Lower traces in each set are recorded from the dorsal root entry zone.) Records A–D and G show the EPSPs evoked by two different strengths of stimulation of the plantar cushion. The strengths are indicated to the left in multiples of the threshold value for evoking EPSPs in the plantar motoneurones. Records G–I were all taken with the same pad stimulus — H and I during passage of current through the microelectrode. Records J and K were taken with faster sweep and higher amplifications to illustrate the central latency of the excitatory actions from the pad. Note separate calibrations for these records. All records consist of many superimposed traces. The amplitude calibrations have reference only to the intracellular records.

depolarizations of the cells were regularly carried out in order to exclude misinterpretations of reversed IPSPs due to chloride leakage from the electrode. In spite of this no inhibitory components have been detected in the synaptic actions evoked by moderate pad stimuli in plantar units. But the pad stimulus was usually not raised above 4 times threshold for effect in the most sensitive plantar motoneurones to avoid firing of the motoneurones and it can therefore not be excluded that stronger stimuli may cause inhibition.

Inhibition of extensor motoneurones (Fig. 11 E) and excitation of flexor motoneurones (Fig. 11 F) were regularly evoked by pad stimuli but invariably the threshold for this effect was higher than for the EPSPs in plantar motoneurones. The effects from the pad to PBSt or G S motoneurones were not different from those which were received from many other skin areas or from FRA in general.

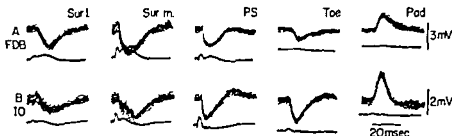


Fig. 12. *Intracellular recording as in figure 11 from a flexor digitorum brevis (A) and an interosseus motoneurone (B) showing synaptic actions by volleys in different skin afferents.* Inhibition is evoked from the medial and lateral sural nerves, from the superficial peroneal nerve and by stimulation of a peripheral toe pad (toe). As in figure 11 stimulation of the plantar cushion (pad) gives excitation.

Effects from other skin afferents

This part of the investigation was limited to stimulation of the medial and lateral sural nerves, the superficial peroneal nerve (deprived of its two muscular branches) and any one of the peripheral toe pads. This was done in parallel with the analysis of effects from the other FRA. On the whole all these afferent systems gave dominating inhibitory actions in the plantar motoneurones. However, the synaptic actions from skin afferents often appeared to be of a mixed type swinging the membrane potential between depolarization and hyperpolarization. In 25 % of the cells the synaptic response was a pure hyperpolarization. Even in some of these cells the passage of polarizing currents through the recording electrode revealed components of excitatory synaptic actions.

Fig. 12 gives typical examples of the units showing only inhibition from the FRA. The upper records were taken from a FDB motoneurone identified by antidromic stimulation of its axon in one of the nerve branches to this muscle. The lower records are from another motoneurone with its axon in a deep branch to the interossei. The IPSPs evoked by the different skin afferents correspond very well to those obtained by earlier investigators (ECCLES *et al.* 1959a) in motoneurones of hindlimb extensors and to the IPSPs in many of the gastrocnemius motoneurones encountered during the present experiments. The shortest central latency of these IPSPs is 2.5—3 msec and they have a fairly rapid rise when evoked in the skin nerves. The IPSPs evoked by electrical shocks to the toe pad, like the EPSP from the central pad, have slightly longer latencies ranging from 3 to 4 msec and a longer rising time. However, this need not indicate a longer interneuronal chain (cf. p. 28). The central pad and the toe pad

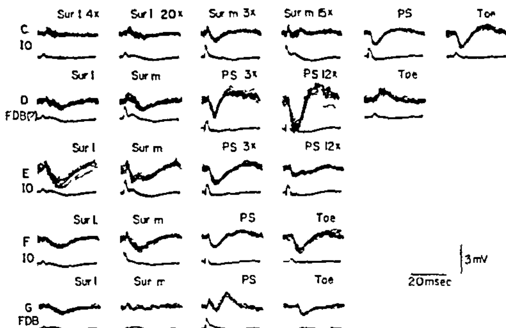


Fig. 13. *Intracellular recording as in figure 11 from several motoneurones of plantar muscles showing different synaptic actions by volleys in skin afferents.* When different stimulus strengths have been used, these are indicated in each record in multiples of threshold for the nerve.

stimuli were delivered through similar electrodes inserted in the same way and it is interesting to note the resemblance between the synaptic actions despite their opposite sign. There was a slight threshold difference, the toe pads needing 20—100 % higher stimulus strength.

Fig. 13 is representative of the major part of the investigated plantar units. The records were all taken with citrate electrodes and all except those at G from the same experiment. As is seen, the dominating effect is inhibition. Several of the records illustrate the common finding that the beginning of the postsynaptic potentials are excitatory, sometimes discharging the cell, and followed by more or less pronounced IPSPs (sometimes this reverses again to a late excitation as from PS in units D and G). This initial phase of excitation was most commonly evoked from the medial sural nerve, as in the figure.

The axons of units C, E and F were stimulated antidromically in the deep branches to the interossei, unit G was innervating FDB, whereas unit D (receiving the largest Ia actions from FDB) was lost before its axon had been localized below the bifurcation of the tibial nerve in the foot. It is not surprising that excitatory actions could be traced to the sural

nerve since it innervates skin down to the heel and in some cases part of the proximal plantar skin belonging to the area in which adequate stimuli could activate these muscles. PS innervates the sides of the toes and foot dorsum and will sometimes contain afferents even from plantar areas of the toes (LANGLEY 1923). Some excitatory actions from this nerve were seen to mix with the inhibitory in about 5 % of the analysed units. A few of these could be identified and they belonged to FDB or L. This may be related to some findings in the experiments with monosynaptic testing that indicate a difference between FDB and IO in receptiveness to toe stimuli. The monosynaptic test reflex evoked in the tibial nerve and recorded in the FDB muscle was facilitated in some cats from the toe pads whereas this was never the case with IO test reflexes recorded in the muscle or with compound test reflexes recorded in ventral roots. Synaptic actions from the toe pads were not systematically tested in all the experiments with intracellular recording. However, in the four cats where toe pads were stimulated only two plantar motoneurones out of 25 were excited. One of these was antidromically identified to be an FDB unit, the other received its largest Ia inflow from FDB (unit D, Fig. 13).

Different fibre groups within the same nerve were sometimes demonstrated to give different actions as in unit A from Sur m. and in unit C from PS, where increased stimulus strengths added some excitatory action (cf. the effects from muscle afferents Fig. 14).

It has not been possible to correlate further the variations in receptiveness from skin afferents with the different muscles innervated by the plantar motoneurones. Ten of the cells have been identified to innervate FDB or L and 25 identified to have axons in the deep branch of the lateral plantar nerve. Among the latter, two cells could be traced to IBV and a few others likewise left the deep branch early, possibly innervating the adductors or even FDB (the lateral portion of which may receive a very thin nerve from this branch). The rest of this group had axons entering the deep interosseus musculature. No systematic difference in skin receptiveness was observed between the FDB motoneurones and those of the interossei, nor was the variability of the synaptic actions less in either of these groups than in the larger group of unidentified plantar units.

Effects from joint afferents

For practical reasons the posterior nerve to the knee joint was chosen as representative for joint afferents. No evidence has been presented for synaptic actions in motoneurones by the larger joint afferents, the flexor

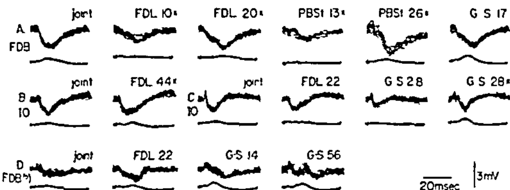


Fig 14 Intracellular recordings from the same plantar motoneurones as in figures 13 A B and 13 C D illustrating the synaptic actions by volleys in high threshold joint and muscle afferents The stimulated nerves and the stimulus strengths in multiples of nerve threshold are given for each record

reflex action is evoked in fibres of the diameter group between 2 and 7 μ according to Eccles *et al* (1959a)

Inhibition of plantar motoneurones is more predominant from high threshold joint afferents than from the skin Large IPSPs as illustrated in the first record of Fig 14 were seen in most of the cells and there was no difference in this respect between cells identified as FDB L or IO motoneurones A similar effect was found in G S motoneurones as has been described by Eccles *et al* (1959a) However very slight signs of an initial EPSP from the joint nerve are not uncommon (see unit B of Fig 14) and in a few cases they have been as discrete as that of cell C Fig 14 Such EPSPs appeared more frequently among motoneurones receiving excitatory actions from the other FHA like cells C and D (The motoneurones of Fig 14 are the same as those marked with the corresponding letters in Fig 13)

In one experiment an analysis was performed of the early EPSP evoked in the joint nerve This phenomenon has been observed also in some G S motoneurones and one of these and one IO unit were recorded from with carefully graded stimulation of the joint nerve (Fig 15 A B) Immediately after these sequences the records from the sciatic nerve (Fig 15 C) were taken *showing the fibre groups activated by the different stimulation* strengths The threshold of the joint nerve was checked at intervals during the intracellular recording and found to be constant At about 1 6× threshold for the largest fibres a second group is activated this potential is seen to increase to about 1 9× and is then followed by activity

in the high threshold fibres which are not quite maximally stimulated at 3.4×. In agreement with earlier investigations no effect is seen in the motoneurones from volleys in the largest fibre group. Since the IPSPs evidently appear at stimulus strengths about 1.6× and attain a maximal size below 2.2× threshold it is concluded that they are caused by activity in the second subdivision of fibres recorded in the sciatic nerve. The inhibitory action comes with the smaller fibres at about 2× threshold likewise in agreement with the investigation referred to above.

Effects from high threshold muscle afferents

To obtain effects from group II and III muscle afferents the nerves to G S and I DI were stimulated. (The branch to the interosseus membrane was removed from the I DI nerve.) The use of these muscle nerves or one of them alone was deemed sufficient since ECCLES *et al.* (1959 a) demonstrated very wide and homogenous receptive fields for actions to motoneurones by high threshold muscle afferents. Other muscle nerves were tried in a few experiments (cf. Fig. 14 A) but their actions were the same.

The muscle afferents like the joint afferents give more pure inhibitory actions than the skin afferents but a parallelism between the effects from skin, muscle and joint can be noted. In the units receiving inhibition from the skin IPSPs were always seen on stimulation of other FRA, whereas some of the units receiving mixed skin actions could get a small initial IPSP or display signs of later excitatory actions from the joint and muscle afferents. The excitation was never seen to dominate the whole synaptic action from the latter afferents. Fig. 14 A and B show records from an FDB motoneurone and an IO motoneurone respectively, both with marked IPSPs evoked from all the afferent sources tested (cf. Fig. 13). The stimuli of the muscle nerves were for the records in A of two strengths. Since the lower strength was supramaximal for group II afferents the additional effect of the stronger stimulus must be due to impulses in group III afferents. IPSPs evoked by impulses in group II afferents are illustrated in some records of Fig. 18 where the stimuli have been kept subliminal for group III. (The ventral roots were cut in this experiment.) About 80 % of the investigated plantar units received similar synaptic actions, i.e. of a kind that could be expected in extensor motoneurones according to the work of ECCLES *et al.* (1959 a). The remaining cells displayed varying patterns of synaptic activity as represented by neurones C and D in Fig. 14. Signs of excitation were sometimes revealed

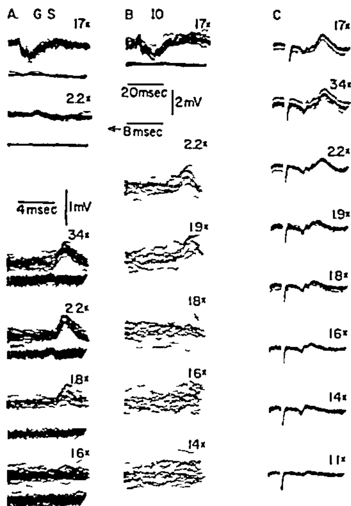


Fig 15 *Intracellular recording from a gastrocnemius soleus (under A) and an interosseus motoneurone (under B) showing synaptic actions from knee joint afferents of different diameter* KCl filled microelectrodes were used The records under C show action potentials in joint afferents recorded from the sciatic nerve The joint nerve was stimulated with the strengths indicated in each record in multiples of threshold value Calibrations have reference only to the intracellular records (20msec/2mV to the uppermost records 8 msec/2mV to the second record under A and 4 msec/1mV to the other records)

when the stimulus strength of muscle afferents was raised within the group III range This is illustrated for G S afferents in both the C and D units In C at weak stimulus strength the small Ia EPSP is followed by a marked IPSP When the stimulus strength is increased not only the later inhibition increases with it but there appears a small EPSP in the

beginning, indicating the existence of excitatory pathways from finer afferents. In unit D, which was exceptionally rich in excitatory inflow from the FRA, even more pronounced alterations in the synaptic actions from G.S. afferents occur when the stimulus includes the finest fibres. In the record taken with the lower stimulus strength the postsynaptic potential has a somewhat dentate shape in the first part. This was seen in single sweeps to be due to a rapid irregular swinging of the potential 7 or 8 times during about ten milliseconds, indicating asynchronous competitive synaptic actions on the cell. In the superimposed records shown these digressions cover each other resulting only in a broadening of the trace.

The experiments were performed both with the ventral roots intact (to permit antidromic identification of the units) and with the L_6, L_7, S_1 and S_2 ventral roots sectioned. In the former group of experiments recurrent inhibition must have been produced from the antidromic volley in α efferents (ECCLES, FATT and KOKETSU 1954; BROOKS and WILSON 1959; WILSON, TALBOT and DIECKE 1960; RENSHAW 1941; WILSON 1959). This makes it difficult to judge the origin of actions evoked by stimuli of low threshold group II strength. However, the FRA actions considered in those experiments were those evoked at strengths above maximal for α efferents.

Many of the motoneurones impaled could not be identified with respect to the particular muscle they innervated in the foot. It is true that the synaptic actions from the different sources discussed in this chapter have been very similar in identified motoneurones of different muscles but quantitative differences regarding the excitatory effects cannot be excluded. There are indications of such differences in the results of experiments with monosynaptic testing. In a few cases facilitation was seen of FDB test reflexes when joint or muscle nerves were stimulated. Like the facilitation from toe pads discussed on page 32 this was never seen with test reflexes recorded in IO muscles or in ventral roots. However in the majority of these experiments there was inhibition also of FDB from these FRA. Test reflexes to G.S., FDL or PI were also conditioned for comparison but with them only inhibition was seen from joint and high threshold muscle afferents.

Effects from group I muscle afferents

a) Ia excitation

The present conclusions about intrinsic Ia connections of the foot muscles are mainly based upon data from 41 plantar motoneurones encountered in four experiments, but they are supported by findings during the experiments related earlier in this chapter. No systematic data con-



Fig 10 Intracellular recording as in figure 11 from an interosseus motoneurone showing large monosynaptic EPSP from interosseus (IO) afferents but very little effect from flexor digitorum brevis (FDB) or lumbrical (L) afferents The notch in the rising phase of the EPSP from IO is due to the field potential from firing neighbouring motoneurones as shown by the extracellular record to the right in the figure The long distance from the stimulation point in the foot causes a delay of the antidromic impulses in the motor axon in relation to the Ia afferent volleys The antidromic action potential therefore invades the cell comparatively late as seen in the second record taken at threshold for the motor axon The third record was obtained with a higher stimulus repetition frequency (note also different amplification) and shows the separation in IS and SD components of the antidromic spike

cerning Ia actions from the plantar muscles could be obtained in these earlier experiments since the dissection of the nerve branches to the plantar musculature was difficult if the innervation of the pads was to be kept intact Even if this innervation is sacrificed it is difficult to mount all nerve branches in the foot separately for stimulation The analysis has thus been restricted to the following combinations of muscle nerves 1) FDB+L the branches from the medial plantar nerve that innervate *the medial parts of FDB and the lumbricals 2) IO* the branches from the lateral plantar nerve innervating the interossei (leaving out FBV) and the adductors 3) FDB lat + FBV the remaining muscular branches of the lateral plantar nerve In most cases however the effects from this group were estimated by a comparison of the effects from the whole *lateral plantar nerve with those of the IO branches The FBV was grouped* with FDB instead of with the interossei because of its mechanical function (see chapter III) Since only two of the experiments were made with intact ventral roots many of the motoneurones were not identified antidromically The Ia EPSPs recorded with stimulation of different nerves have been expressed in per cent of the maximal Ia EPSP evoked from the tibial nerve stimulated shortly above the ankle

The plantar motoneurones were found to form two distinctly separate groups with respect to their Ia receptiveness Two thirds of the cells

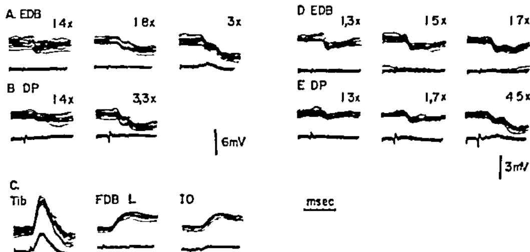


Fig. 18 *Intracellular recording as in figure 17 from two plantar motoneurones (A—C and D—E respectively) illustrating the disynaptic inhibition by group Ia afferents in the nerves to extensor digitorum brevis (A, D) and the pretibial flexors (B, E).* Stimulus strengths are given in multiples of nerve threshold. At C are shown the Ia EPSPs evoked from the tibial nerve and from the plantar nerve branches. Approximately 60 % of the Ia excitation comes from the medial nerve branch to the superficial plantar musculature.

was monosynaptically excited from G S and only one during the FRA experiments (see Fig. 14 C).

The Pl nerve was only stimulated in one experiment and it gave no Ia excitation in the ten plantar motoneurones impaled. This is surprising since Ia effects from Pl to EDB motoneurones were reported by Eccles *et al.* (1957 b).

b) Ia inhibition

It is to be expected that Ia volleys from the antagonist muscles would exert disynaptic inhibition upon the plantar motoneurones. This has been recorded in many of these cells upon stimulation of low threshold group I afferents of the nerves from the pretibial flexors and EDB. Fig. 18 demonstrates two of the cells with large Ia IPSPs from both of these sources. No attempt has been made to disclose the separate contributions from EDL, TA and the peroneal muscles. There was no evidence of any difference in Ia inhibitory actions to the two groups of plantar motoneurones.

c) Ib actions

The actions of Ib impulses in the EDL, GS, PL and DL nerves on plantar motoneurones have been investigated but not the autogenetic inhibition from plantar muscles. The EDL nerve was most frequently used as it is known to give particularly large Ib inhibition in extensor motor nuclei (ECCLES *et al.* 1957c). This was true also for the motor nuclei of the different plantar muscles, as illustrated in Fig. 17 P. The incidence of Ib inhibition was much higher than that of Ia excitation from this nerve and the Ib effects were often largest when both were present. The time course in the illustrated records, with a latency to the onset of the IPSP of nearly 3 msec, indicates a trisynaptic pathway. This was the usual finding and conforms well with the results of ECCLES *et al.* (1957c). The other extensor nerves were only stimulated occasionally at strengths suitable for an evaluation of Ib actions. Similar IPSPs were then recorded and it seems that the receptiveness is of the type that has been reported from the investigations of other extensor motor nuclei.

Ib excitation from the antagonist pretibial flexors was not found in the plantar motoneurones. Alterations in the time course of the Ia IPSP from DP or EDB nerves, when the stimulus was increased in the Ib range, could never be established with certainty (Fig. 18). Large inhibitory group II effects often occurred with a stimulus threshold close to that of the Ib afferents and this made the evaluation of effects from maximal Ib stimulation difficult. However, it is evident from the records that any Ib effects from the DP and EDB nerves must be very small in the plantar motoneurones.

TOE FLEXORS

The reflex connections of the antagonists EDB and EDL have been studied by monosynaptic testing and not with intracellular recording. In all of the six experiments in which the EDB monosynaptic reflex could be used it was facilitated from the FRA. The different types of afferents were equally effective. It was not possible to evoke inhibition of the EDB test from any of the conditioning nerves and, as reported in chapter IV, not even stimulation of the plantar skin gave inhibition, which would have been expected according to HAGBARTH (1952).

The EDL nucleus likewise proved to receive excitatory actions from the FRA (in 5 experiments). However, some exceptions to this were seen: adequate stimulation of the foot dorsum was tried in two cats and inhibited the EDL test reflex, particularly when the more lateral skin regions were pinched. In one of these cats inhibition could also be evoked by pinching of the lateral part of the planta. Stimulation of the joint nerve

elicited a mixed response in this cat: early facilitation followed by inhibition. In another animal volleys in the joint nerve inhibited the EDL test to about 50 per cent without any signs of facilitation (relatively strong electric shocks to the central pad had the same effect, cf. chapter IV — no other conditioning afferents were tried in this experiment). The FRA actions to TA were sometimes tested for comparison with EDL and the reflex responses were found to be very similar for the two motor nuclei, effects from the foot skin included.

DISCUSSION

The experiments with intracellular recording from motoneurones have confirmed the results reported in the last chapter that the intrinsic plantar muscles FDL and PI receive excitatory actions from pad afferents whereas there is no trace of such actions to the pure ankle extensor G S. It is particularly noteworthy that stimulation of the plantar cushion evoked very large EPSPs in every one of more than 100 impaled plantar motoneurones. The actions to FDL and PI were weaker.

The actions from the pad are in contrast to the inhibitory actions that dominate in the spinal animal with stimulation of cutaneous afferents from other regions and of high threshold joint and muscle afferents. These inhibitory actions from FRA make it possible to conclude that the intrinsic plantar muscles should be classified as extensors of the limb. This is also to be expected according to the investigations of SHERRINGTON (1910) and to later investigations of the crural muscles that act in synergism with the short foot muscles (ECCLES *et al.* 1959 a, HOLMQVIST 1961).

It is true that excitatory actions from the FRA were more often encountered in the plantar motoneurones than has previously been described for extensor motoneurones. However, apart from the excitatory actions from special skin regions (HAGBARTH 1952), it has been reported that extensor motoneurones may receive excitation from the FRA (cf. LUNDBERG 1963) and this has been confirmed in the present investigation since such effects have been found rather often in G S motoneurones. In other investigations in this laboratory large excitatory actions from the FRA have likewise been found in motoneurones of many other extensors.

There are some differences in reflex receptiveness among the motor nuclei of the different plantar muscles. For example FDB motoneurones could sometimes be facilitated from the peripheral toe pads whereas this was never found for interosseus motoneurones. This may indicate a purposeful functional organization in that noxious stimulation of the toes would lead to their retraction by simultaneous contraction of FDB and the toe dorsiflexors (cf. Fig. 2 and GRIMBY 1963). Transmission in such

reflex paths may depend on actions from supraspinal centres which are disconnected from the reflex arcs in the spinal preparation (cf. chapter VI). The existence of these excitatory connections would then only be revealed occasionally in experiments of the present type.

Activity in the interossei would not be profitable for a withdrawal of the toes since they act on the basal phalanges at the plantar side and join the common aponeurosis of the toes dorsally and peripheral to the basal joint (Fig. 2). They will thus produce a toe movement almost opposite to the desired one. On the other hand a noxious stimulus to the more central plantar skin areas should produce reflex activity in both IO and FDB as well as in flexors of the ankle if the foot should be protected and it was shown in the last chapter that the plantar muscles do receive excitatory action from the plantar skin.

The inhibitory response in the EDL and TA nuclei that were elicited in a few cases by lateral plantar stimuli could likewise be a sign of alternative flexor reflex pathways, which under the control of higher centres may make the protective reflexes more elaborate and purposeful. Such alternative pathways have been demonstrated in the cat (cf. LUNDBERG 1963) although in the spinal state the excitatory connections generally dominate to flexors. The variability in receptiveness to FRA actions is particularly evident for the pretibial flexors (ECCLES *et al.* 1959 a, GRAHAM BROWN and SHERRINGTON 1912, PAINTAL 1961 and MECIRIAN 1962). SHERRINGTON (1910) found in the spinal monkey that stimulation of the lateral plantar skin gave eversion of the foot plus ankle flexion, whereas stimulation of medial skin areas gave inversion of the foot. It is possible that similar reflex organizations exist in the cat. This would account for the frequency of FRA inhibitory actions to ankle flexors, which now and then leads to a dominance over the excitation even in the acute spinal state.

On the whole, however, there are not many indications of such purposeful patterns of the protective reflexes in the spinal cat. The experiments in humans by KUGELBERG and others referred to in chapter VI give a much more elaborate picture of the protective reflexes, but it should be recalled that they were done on intact humans with possibilities for descending control systems to select alternative reflex pathways (cf. HOLMQUIST and LUNDBERG 1961, LUNDBERG and VOORHOEVE 1962).

The EDB nucleus is regularly facilitated in the flexor reflex whether this is evoked from the skin of the foot or from any other source. This is a strong support to the classification of this muscle among the flexors which was suggested already by SHERRINGTON (1910). HAGBARTH (1952) could evoke facilitation of the EDB monosynaptic reflex by stimulation of skin only from the foot dorsum — the remaining surface of the limb giving inhibition, especially the plantar cushion. However, as

his experiments in this case were performed on decerebrate cats there may have existed a strong influence upon the flexor reflex arc from supraspinal centres It has been demonstrated (ECCLES *et al* 1959 b HOLMQUIST *et al* 1961) that in the decerebrate state the transmission in the flexor reflex pathways is depressed and that the control of inhibitory pathways can be released separately by pontine lesions (or even accidentally e g by a shortlasting blood pressure fall) leaving the supraspinal depression of excitatory pathways intact This situation with a partial control of reflex mechanisms is not uncommon in decerebrate preparations either due to spontaneous variations in the activities of the supraspinal centres or to the operative technique and could explain the discrepancies between HAGBARTH's results and those obtained in this work

The plantar muscles resemble the more proximal extensors of the limb also in other respects The inhibition exerted by Ib afferents and the weak excitatory action from low threshold joint afferents are both characteristics of extensors These particular excitatory effects that a volley in the low threshold joint fibres may have in extensor motoneurones have not been reported earlier possibly due to the fact that they often are very small and cut off by the much larger inhibitory effects The inhibition might appear with only a slight increment of stimulus strength and before the EPSP is maximal The two fibre systems may have an overlap in the diameter spectrum in which case the larger inhibitory effects will conceal the excitatory ones except when the stimulus is kept just supraliminal for the excitatory fibre group Such a submaximal stimulus would not produce any effect visible above the random fluctuations of the membrane potential

The larger afferents from the knee joint are connected to 3 different types of receptors identified by SKOGLUND 1956 In the diameter range between 5—15 μ fibres emerge from Golgi endings from Ruffini endings and also from a few Pacinian corpuscles The Golgi endings are fewer than the Ruffini endings and their fibres are the largest The small action potential coming with the lowest stimulus threshold is probably propagated in fibres that belong to the Golgi endings and it has no visible effect in the motoneurones The sudden appearance of the EPSP with the excitation of a few slightly smaller fibres in the nerve could fit with an assumption that fibres from the Pacinian corpuscles are responsible This is further supported by the existence of similar excitatory actions in motoneurones evoked by stimulation of nerve fibres from Pacinian corpuscles located in the interosseus membrane between tibia and fibula (LUNDBERG personal communication)

The Ia excitatory actions to motoneurones of plantar muscles are largely confined to intrinsic connections in the foot Only relatively small Ia EPSPs were sometimes recorded with stimulation of the nerve

branches to the long toe extensors. Volleys in the Ia fibres from pretibial flexors produce the expected reciprocal Ia inhibition. The relatively restricted analysis that could be performed on the intrinsic Ia actions between the plantar muscles further shows that the deeper musculature receives very little Ia excitation from the superficial muscles. In the other direction the heteronymous Ia effects are generally larger. The significance of this is not clear. It adds to the differences in reflex receptiveness between these two muscle groups that have been discussed earlier in connection with the FRA actions.

The organization of Ia connections to motor nuclei of hindfoot muscles is thus in agreement with the concept of the myotatic unit (LLOYD 1946 b). This is in contrast to the more complex Ia connections to motoneurones of hip and knee muscles (ECCLES *et al.* 1958) and of forefoot muscles (SCHMIDT and WILLIS 1963).

SUMMARY

Intracellular recording has confirmed the existence of an excitatory reflex path from the plantar cushion to all muscles that plantar flex the toes. Large EPSPs were evoked by pad stimulation in all the investigated motoneurones of plantar muscles as well as in FDL and PL motoneurones. Stimulation of toe pads generally evoked IPSPs but EPSPs were seen in some EDB motoneurones.

The motoneurones of plantar muscles receive mainly inhibitory actions from group II and III muscle afferents, high threshold joint and cutaneous afferents. Hence the plantar muscles should be classified as physiological extensors. However, mixed excitatory and inhibitory FRA actions were found on many of these motoneurones, particularly from skin afferents but also from muscle afferents.

A small EPSP was evoked in some IPM and G S motoneurones from low threshold joint afferents.

Motoneurones of the deep plantar muscles receive little heteronymous excitation from Ia afferents, those of superficial muscles have a wider Ia receptive field in the foot. Very little Ia excitation is exerted by afferents from crural muscles. Ia afferents in the DP and EDB nerves cause disynaptic inhibition.

The motoneurones of plantar muscles, like those of other extensors, are synaptically inhibited by Ib afferents from extensors. No effect was found from Ib afferents of antagonist flexors.

Monosynaptic testing showed only excitatory actions from the FRA to the EDB motor nucleus. This supports the classification of EDB as a physiological flexor.

Chapter VI

Supraspinal control of the toe extensor reflex

INTRODUCTION

Transmission in spinal reflex paths to motoneurones is strongly influenced from higher centres. In the decerebrate animal the transmission of flexor reflexes is strongly depressed by tonically active centres in the brain stem (ECCLES *et al.* 1959 b, HOLMQUIST *et al.* 1961). This is true both for the excitatory and the inhibitory pathways although different regions in the reticular formation seem to be responsible for the depression of them. There is also evidence of inhibition from the brain stem through primary afferent depolarization (CARPENTER, ENGBERG and LUNDBERG 1962). On the other hand there may be spinal reflexes that require activity in descending control systems for their appearance. Experiments with administration of L-3,4-dihydroxyphenylalanine in the spinal cat have given evidence of such reflex paths that are normally concealed in the spinal state (ANDÉN, JUKES, LUNDBERG and VYKLICKÝ 1964). In the intact animal cortical impulses descending in the pyramidal tract enhance the synaptic actions in motoneurones of group Ia (inhibitory actions), Ib and flexor reflex afferents (LUNDBERG and VOORHOEVE 1962, LUNDBERG, NORRSELL and VOORHOEVE 1962). These regulating mechanisms have been suggested to depend on synaptic actions in interneurones of the reflex arcs.

DECEREBRATE CONTROL

Five experiments were performed on decerebrated cats and their reflexes were then investigated both before and after transection of the spinal cord. The toe extensor reflex was never completely extinguished in the decerebrate state although it was markedly depressed. It could always be elicited with electrical stimuli but in a few cases not with adequate stimuli. Compared to the response in spinal animals (Fig. 3, chapter IV) the effect of pad pressure was very small and shortlasting, almost disappearing in one or two seconds. A spinal transection at Th₁ gave a prompt release of the reflex within a few minutes. No attempts were made

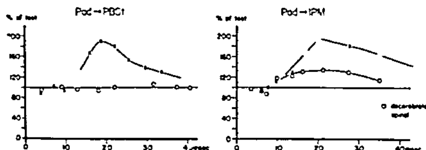


Fig 19 *Excitability changes in the motor nuclei of posterior biceps semitendinosus (PBSt) and of the intrinsic plantar muscles (IPM) following electrical stimulation of the plantar cushion in the decerebrate (O) and spinal (X) cat* Monosynaptic testing as in figure 6 The excitation of the IPM nucleus is not completely depressed in the decerebrate state

to localize the inhibitory centre nor its descending pathway The cerebellum was not removed

A comparison was made between the decerebrate control of reflexes from the pad to a flexor (PBSt) and to the plantar muscles It was not possible in the animals with good reflex control to obtain any change in the testing monosynaptic reflex of PBSt upon electrical stimulation of the pad even if this stimulus was several times stronger than needed to facilitate the IPM test (Fig 19) The spinal transection released the PBSt facilitation and gave an increase in the effects to IPM

EFFECTS FROM THE SENSORIMOTOR CORTEX

In an investigation of plantar reflexes it was of special interest to evaluate the cortical influences upon the reflex pathways An important question was whether the toe extensor reflex could be facilitated by corticospinal impulses travelling in the pyramidal tract Experiments were performed on lightly anaesthetized cats (Nembutal) with intact brains where the sensorimotor cortex was exposed for stimulation (see methods) The animals were not paralysed as in other experiments and the cortical stimulation therefore always produced vigorous movements if it was raised to a sufficient strength The stimuli employed for conditioning were invariably too weak to produce any movements in the animal

Cortical stimuli applied within the shaded area of Fig 20 J have a polysynaptic excitatory action in the motor nuclei of plantar muscles Fig 20 A and B show the facilitation of a monosynaptic test reflex to FDB (recorded in the muscle) following a short train of cortical stimuli The same effect is obtained with a compound test reflex of IPM recorded in

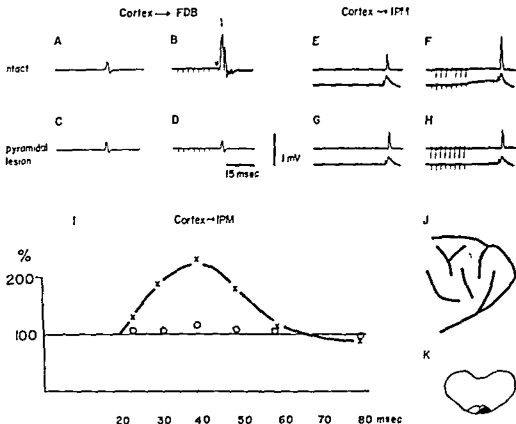


Fig 20 *Cortical actions in the motor nuclei of the intrinsic plantar muscles* Monosynaptic test reflexes of flexor digitorum brevis alone (A) and of all the plantar muscles (E) are facilitated (B F) by a train of conditioning stimuli delivered to the sensorimotor cortex (The cortical stimuli used did not evoke any motoneuronal discharge) The effect is abolished after transection of the pyramid (C D G H) The curve at I shows the time course of the cortical facilitatory action in the plantar motor nuclei (× =intact O=transected pyramidal tract) J and K illustrate the areas of cortical stimulation and pyramidal lesion respectively The FDB test was recorded in the muscle and the IPM test in the ventral root The cord dorsum potentials are included in records E—H (lower traces) All records consist of several superimposed traces

the ventral roots (Fig 20 E F) (This is in contrast to the inhibitory action exerted upon other extensor motor nuclei Even FDI and PI are inhibited by the cortical stimulation) Interruption of the pyramidal tract abolishes the facilitation (C D and G H respectively) The lesion was made at the pyramid which had been uncovered at the skull base during the preparation of the animal so that only the dura remained intact A histological control of the extent of the lesion was made after each experiment (cf drawing in Fig 20 K) The curves in the lower part of the

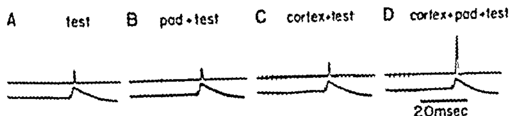


Fig. 21 *Spatial facilitation between reflex actions from the plantar cushion and descending cortical actions upon the motor nuclei of the intrinsic plantar muscles* Monosynaptic test reflexes of the plantar muscles recorded in the ventral root (A) are moderately facilitated by a weak conditioning shock to the pad (B) A few of the many superimposed traces show unstable facilitations to about 150 % But most of the traces have about the same amplitude as the test The cortical stimulation was adjusted to give a minimal effect in itself (C) and the two conditioning stimuli thereafter combined giving large facilitation (D)

figure illustrate the time course of the cortical actions in plantar motor nuclei

In order to ascertain whether this cortical excitation or at least part of it could be due to an activation of interneurones in the toe extensor reflex arc a combined conditioning was performed The cortical stimulus and an electrical conditioning stimulus to the plantar cushion were first adjusted to evoke a liminal facilitation of the monosynaptic test when given individually Both stimuli were then combined and found to produce a very good facilitation This is illustrated in Fig 21 where the test increase in record D is much larger than the sum of the liminal facilitations in B and C This gives evidence for a convergence of the impulses from cortex and pad into a common pathway to the plantar motoneurones

DISCUSSION

The experiments have shown that there is a very effective tonic depression of the toe extensor reflex in the decerebrate state In several experiments strong pressure on the pad had no effect on plantar motoneurones However even in cats with very effective tonic control of the flexor reflex electrical stimulation of the pad usually gave excitation of plantar motoneurones This finding cannot be taken to indicate a differential control of the two reflexes the explanation may be a difference in synaptic linkage Furthermore facilitatory actions from low threshold cutaneous afferents to other extensor motoneurones are invariably present in the

decerebrate state (e.g. the excitation of G S motoneurones by volleys in the sural nerve)

The cortical excitation of the motor nuclei of plantar muscles is in contrast to the inhibition of other extensor motor nuclei that has been reported earlier (LUNDBERG and VOORHOEVE 1962) and also confirmed during the present experiments. LUNDBERG and VOORHOEVE postulated that the action from the pyramidal tract on motoneurones is secondary to excitation of interneurones of reflex paths and correlated the inhibitory action from cortex to extensor motoneurones with the fact that the inhibitory path from the FRA is the dominating spinal reflex pathway to extensors. The finding of excitatory actions from cortex to plantar motoneurones should be correlated with the very effective excitatory reflex path from the pad and with the findings suggesting that the cortical effect is exerted on the interneurones of this path.

The results of these experiments are of particular interest in relation to the work that has been done on human plantar reflexes. The nociceptive plantar reflexes in man are organized in a purposeful manner to withdraw the stimulated area from the stimulus. This implies that stimuli to the ball of the big toe give a flexor reflex with dorsiflexion of the toe and foot whereas stimulation of the ball or hollow of the foot elicits plantar flexion of the toes (WALSHE 1956, EKLUND, GRIMBY and KUGELBERG 1959, KUGELBERG, EKLUND and GRIMBY 1960, GRIMBY 1963). This reflex pattern is dependent on a normal function of the pyramidal tract and lesions of this tract will lead to the appearance of the well known *phenomene des orteils* of BABINSKI (1896). It has been suggested that impulses in the pyramidal tract regulate the transmittability in the alternative reflex pathways providing means for an integration of flexor and extensor action. The cerebral influence on the plantar reflexes in man is demonstrated by the experiments of GRIMBY (1963) where he shows that the normal spinal reflex response to an unexpected electrical plantar stimulation may be reversed if the subject, before he receives the stimulus, is concentrating his thoughts on making a certain movement of the toes (without actually contracting any muscle). The susceptibility of human spinal reflexes to hypnotic suggestions (HAGBARTH and FINER 1963) further illustrates the capability of this cerebral control.

It is difficult to know to what extent it is justifiable to draw parallels between reflexes from the foot sole in man and in cat. The descending control of plantar reflexes in man is regarded as important for the regulation of reflex movements for protective purposes. In all likelihood the toe extensor reflex is not part of a protective reflex and the cortical control of it has probably a more direct significance in the motor performance of the animal than a regulation of a protective reflex would have

SUMMARY

There is an effective tonic depression of the toe extensor reflex in the decerebrate state: the reflex cannot be elicited by natural stimuli. Electrical pad stimuli usually have some excitatory effect on plantar motoneurones even in the decerebrate state.

Impulses from the sensorimotor cortex travelling in the pyramidal tract give excitation in plantar motoneurones in contrast to the inhibition of other extensor motoneurones. This particular effect in the IPM nuclei may be due to an activation of interneurones in the toe extensor reflex arc since there is spatial facilitation between the cortical effect and the excitatory actions from the pad.

Chapter VII

Participation of foot muscles in stepping

INTRODUCTION

The physiological activity of hindlimb muscles during locomotion has been studied both in man (EBERHART and INMAN 1951, Prosthetic Devices Research Project 1953) and in cat (ENGBERG and LUNDBERG 1962, ENGBERG 1962). In the cat this was done in an attempt to evaluate the importance of certain reflex mechanisms to the normal locomotion. It was established by ENGBERG *et al.* (1962) that different hip, knee and ankle extensors are similarly activated during the step cycle. Their main activity comes during the stance phase (cf. Fig. 22) of the step, often with a very sudden onset immediately before the foot is put down. This activity is preceded, however, by a weaker activity during what PHILIPPSON (1905) termed the first extension phase, when the flexed limb has been swung forward and is extended towards the ground. The flexors are generally also active during this period, as shown by ENGBERG (1962). They seem to balance the extensor contraction when the foot is placed and are then relaxed during the second extension phase to be activated again shortly before the beginning of the flexion phase. Records from these earlier investigations have been included in Fig. 22. The extensors are represented by the vastus lateralis part of quadriceps (Q) and the part of semimembranosus that inserts on the femur (Sm), the flexors are represented by semitendinosus (St) and TA. There is some difference between the activity patterns recorded during fast and slow moving, particularly in the flexor muscles that are less active in the first extension phase during slow walking.

NORMAL ACTIVITY IN FOOT MUSCLES

In the *plantar musculature*, recording was made from both the deep and the superficial layers. The method of electrode localization was not considered reliable in the smaller plantar muscles close to the FDL tendon and recording was therefore only made from some interossei and from the different parts of FDB.

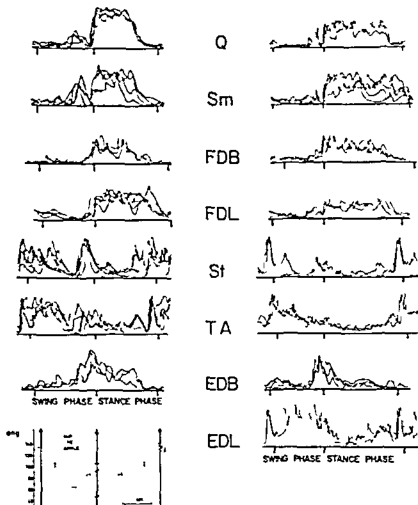


Fig. 23 *Integrated electromyograms from a variety of hindlimb muscles illustrating their activity during locomotion.* Each muscle was recorded from (cf. fig. 23) during several step cycles and a number of the "integrated" records selected and superimposed (see methods). In the left row are records taken with the cat running relatively fast (trot), in the right are records from slower pacing. The amplitudes of the different records are not comparable. Bottom left is a set of curves measured from a moving film showing the angular movements in the hip, knee and ankle joints. The arrows indicate the moments when the foot is lifted from the ground (↑) or put down (↓). These moments are likewise indicated by marks under each set of records. Swing phase thus denotes the period during which the foot is lifted and swung forward, stance phase the period when the foot is in contact with the ground.

Fig. 23 illustrates the type of records that were obtained. There is a great variation in the details of the activity patterns even in subsequent steps of the same speed, therefore several integrated records from dif-

ferent step cycles have been superimposed to give information of the typical activity of the muscles (see methods). The extensor type of activation illustrated for FDB in Fig. 22 was regularly found in all the recording sites. This pattern is, however, slightly different from that of extensors in general since the activity during the first extension phase is very weak. The more or less separate early phase of activity that is typical for hip and knee extensors and common also in some ankle extensors is missing.

FDL was investigated in two cats only. It appears to have a very similar activity to that of the plantar muscles. The early short phase was thus missing in most of the records (see Fig. 22) but sometimes a trace of it was present, coming later than in other extensors and not clearly separated from the following activity.

The electrical activity of FDL and the plantar muscles was found in some records to last longer in relation to the step cycle than is usual for other extensors. Accurate measurement of this difference would require simultaneous recording from the different muscles, since there is a great variability in the duration of activity with different walking speeds. This is most clearly demonstrated by the records from Sm in Fig. 22.

EDL has only been investigated in one cat and the records deviate very little from those of flexors at the hip and knee like gracilis or PBSt. This type of activity is also seen in the synergists of EDL, TA and peroneus longus, which were recorded from during the experiments referred to in the introduction (ENGBERG 1962). All these muscles show a burst of activity at the end of the stance phase when the leg is maximally extended. A greatly varying activity follows in the flexion phase, mostly interrupted by a pause before the first extension phase when the activity is shortly increased again, particularly when the animal moves fast.

EDB displays a very special activity in locomotion. It is activated during the first extension phase like other flexors, but it does not relax immediately after the foot is put down. In some records taken during fast running the activity is nearly as long lasting as that of extensors, but the duration decreases with decrease in speed as illustrated in the figure. The most striking difference between EDB and other flexor muscles is the absence of activity in EDB towards the end of the extension phase and in the early flexion phase.

ACTIVITY IN PLANTAR MUSCLES DURING ANAESTHESIA OF THE PLANTAR CUSHION

Fig. 23 illustrates an experiment with recording from FDB immediately before (A) and some 15—20 minutes after an infiltration of the plantar

A Normal



B Anæsthetized plantar cushion

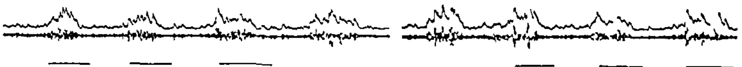


Fig 23 A *Normal activity in flexor digitorum brevis during trot* The upper trace is the electrically integrated electromyogram the lower trace a direct recording The lines under the records mark out the stance phases of the stepping as they have been recorded on the moving film (dotted lines mean that the beginning or end of the stance phase has been estimated approximately)

B *Activity in flexor digitorum brevis with anaesthesia of the plantar cushion* The records were taken 15—30 minutes after those in A and under identical circumstances except for a local anaesthesia of the plantar cushion (see text)

cushion with Xylocain (B). The injection was made so that all parts of the pad including its deeper structures were anaesthetized. 1—2 cc of the Xylocain solution was distributed in small portions within and underneath the pad until it was lifted up and quite extended by the infiltration.

The walking movements of the cat appeared quite normal after this procedure and, as seen from the records of Fig. 23, there was no significant change in the activity of FDB. The recording electrode was left in the same position in the muscle during the whole experiment and its location was as usual controlled afterwards by stimulation.

The interosseus musculature was also investigated with the pad anaesthetized during both fast and slow running, likewise with control records of the normal activity taken immediately before. No change could be distinguished for these muscles either in their activation patterns.

DISCUSSION

The toe extensors differ in activity from other extensors of the limb during the first extension phase of the step. In this phase the extension begins in the knee and ankle joints and to some degree also in the hip joint as was shown already by PHILIPPSON (1905) (see the plotted curves at the bottom of Fig. 22). This agrees very well with the recorded extensor activity. The toes are at the same time lifted up and spread a little so that the peripheral and central pads are put on the ground almost simultaneously. This movement is explained by the early activity in the toe dorsiflexors EDB and EDL and the lack of activity in the plantar muscles during this phase.

The co-contraction of EDB and all the plantar muscles during the early stance phase may be regarded as important for the stabilization of the foot in the step. This view is supported by the anatomical arrangement in the foot. Particularly the deep plantar muscles constitute a system together with EDB that makes the foot very rigid if all the muscles contract simultaneously. EDB is in this system counteracting the adductors and the dorsal parts of interossei II, III and IV, whereas it is synergic with the ventral parts of the interossei at the peripheral toe joints counteracting FDB and FDL.

At the end of the stance phase the cat stretches out the foot and the final push off from the ground is performed by the toes. This would correspond very well with an extra long-lasting activity in the toe extensors which has been indicated particularly in records from FDL. Shortly before the foot leaves the ground the leg is nearly maximally stretched in all its

joints except at the toes The toes are strongly dorsiflexed at their basal joints by the weight of the body and are extended only in the very last part of the extension phase When the toes leave the ground they are at first seen to move in plantar direction and this is explained by the remaining toe extensor activity (the mechanical contraction would outlast the electrical activity with at least some 50 msec) they are only slowly returned in dorsal direction during the flexion phase and the first extension phase It is possible that the strong extension of the leg at the end of the stance phase has importance for the onset of activity in the flexors by giving stretch reflexes in these muscles If so the delayed extension of the toe joints would explain why EDB is not activated simultaneously with other flexors in this phase

The experiments with local anaesthesia of the plantar cushion were performed in order to evaluate the possible role of the toe extensor reflex in stepping In the experiments of SHERRINGTON (1910) with denervation of the feet the natural standing and walking was very little affected Only the toes did not move properly but the motor paralysis produced by the denervation must have been determinant in this In the present experiments there was neither any visible impairment of the walking movements as a result of plantar anaesthesia Even the activation of plantar muscles was apparently unchanged This proves that the toe extensor reflex is not necessary for the activation of these muscles in stepping The experiments do not exclude however that the reflex plays a role in walking since there may be several systems which overlap in the muscular control

SUMMARY

Electromyography has been performed from foot muscles in freely moving cats and correlated with the limb movements

The activation of plantar muscles in stepping mainly follows the pattern described for other extensors of the hindlimb The activity starts shortly before the foot is placed on the ground diminishes towards the end of the stance phase and ceases before the foot is lifted again The toe extensors are less active in the first extension phase than other extensors

EDL is activated according to the normal flexor pattern whereas EDB is not The main activity of EDB is in the first extension phase and no activity is seen in this muscle at the end of the stance phase or in the early flexion phase as in other flexors

In experiments with local anaesthesia of the plantar cushion there was no change in the activation of plantar muscles

General summary and comments

As mentioned in the introduction, reflexes elicited in the foot have commanded much interest in the early reflex literature. Few investigations, however, have been performed on this subject with modern techniques. The present work started with the observation of a plantar reflex that produces toe movements in the cat.

Gentle pressure on the plantar cushion or pinching of the more proximal plantar skin evokes plantar flexion of the toes, particularly in the basal joint. A study of the distribution of reflex actions from the pad, employing adequate as well as electrical stimuli, showed that motoneurones of all the intrinsic plantar muscles and of flexor digitorum longus and plantaris receive excitation. No other extensor muscles of the hindlimb displayed this receptiveness from the planta (Ch. IV). Intracellular recording revealed EPSPs evoked by electrical pad stimuli in flexor digitorum longus and plantaris motoneurones and in all of more than 100 impaled motoneurones of the intrinsic plantar muscles with different function (Ch. V). There was no clearcut evidence of a reciprocal inhibition to motoneurones of the antagonists, the toe dorsiflexors. On stronger stimulation of the pad or any plantar skin area, inhibition is evoked in the motor nuclei of extensors operating at the hip, knee or ankle and usually excitation in the nuclei of flexors, including the toe dorsiflexors (Ch. IV).

Motoneurones of the intrinsic plantar muscles proved to be mainly inhibited by volleys in flexor reflex afferents of different origin in the limb. This implies that the plantar muscles should be classified as physiological extensors of the limb. The antagonist extensor digitorum brevis receives excitation from flexor reflex afferents and is therefore functionally classified as a flexor. The receptiveness of the plantar motoneurones to synaptic actions from group Ia and Ib afferents has also been investigated (Ch. V).

Stimulation of the sensorimotor cortex evoked excitatory action in motoneurones of the plantar muscles in contrast to the inhibition found in other extensor motoneurones. This is explained by the postulate that cor-

ticospinal impulses have excitatory actions on interneurones in the reflex pathway from the plantar cushion to these motoneurones. The effects are mediated via the pyramidal tract and are discussed in relation to the Babinski sign in man (Ch. VI).

In the decerebrate state tonic inhibition is exerted from supraspinal centres upon the reflex pathways from the pad to toe extensors (Ch. VI).

The participation of foot muscles in stepping has been analysed and their activity compared with that of other hindlimb muscles. Flexor digitorum brevis and the interossei are activated similarly to other extensors of the limb, whereas extensor digitorum brevis displays some differences from the usual flexor pattern of activity. There is no marked change in the recorded activity of flexor digitorum brevis or interossei when the plantar cushion is anaesthetized, neither are there any visible changes in the walking movements of the cat (Ch. VII).

In the spinal animal many of the reflex connections from skin to motoneurones seem to have a low degree of spatial specificity and very little is known regarding different actions by afferents from different kinds of receptors. The skin of the plantar cushion is an example of a skin region with specialized connections in that all the toe extensors receive reflex excitation from it. Although pressure receptors in the pad contribute very effectively, it is not certain that the modality specificity is absolute since there is some indication that also other receptor systems may be of importance. However, the excitation of toe extensors evoked by nociceptive plantar stimuli may be part of another (protective ?) reflex system.

The experiments with local anaesthesia of the pad showed that reflexes from this region are not necessary for the activation of plantar muscles in locomotion. This does not exclude, however, that the toe extensor reflex plays a role in the walking of an intact animal since there may be several systems that overlap in the control of muscular activity. The fact that the excitatory actions reach plantar muscles with widely different functions rather indicates that the reflex may serve to stabilize the foot. This function would be of importance both in standing and stepping. The absence of a reciprocal inhibition in the toe extensor reflex increases the possibility of a simultaneous contraction of the plantar muscles and their antagonist extensor digitorum brevis. Such a co-contraction does occur in the beginning of the stance phase in stepping and should further enhance the stability of the foot.

I wish to express my sincere gratitude to professor Anders Lundberg for his great interest in this work and for his invaluable help and criticism. Thanks are due to med kand Staffan Lund for his assistance in some of the experiments and to Mr Erling Eide for construction of apparatuses. Excellent technical assistance was given by Miss Bigmor Fihlgren and Miss Elisabet Kjellberg. Thanks are also due to Miss Helen Brogren, Mr Tomas Palm, Miss Berit Nilsson and Mrs Kerstin Strömwall for help in preparing the manuscript.

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Studies on normal and vagus stimulated hearts

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CONTENTS

PART I

CHAPTER I

Introduction

Already at the beginning of this century the electrolyte content and electrolyte distribution in biological tissues began to arouse attention OVERTON (1902 1904) and HÖBER (1906) studied the mechanism which hindered the transport of sodium chloride from the tissue spaces into the cells and the efflux of potassium phosphate from the muscle fibres They advanced two theories one of which postulated that the fibres were surrounded by a semipermeable membrane which resisted the passage of sugar and certain salts (more permeable for potassium salts than for others) According to the other theory muscle tissue was regarded as a colloidal solution of gelatin and fibrin lacking any enveloping membrane (for a modern similar theory see LING 1962)

FENN *et al* (1934) considered that all chloride in frog striated muscle was extracellular and calculated from the chloride contents of the muscles and plasma the *chloride space* in the muscle (The chloride space in a tissue = the ratio of the chloride content in the tissue to the chloride concentration in the plasma) It was assumed that the interstitial or extra cellular fluid had the same concentration as plasma the chloride space was thus a measure of the extracellular space The distribution of other electrolytes between the cells and the extracellular space was calculated from the chloride space and their concentration in the tissue and plasma

During the last 25 years analytical methods have improved considerably (by the introduction of radioactive isotopes flame photometry spectrophotometry etc.) The notion that the cell is impermeable to ions has been superseded by the concept of a continual exchange of matter between the cell and its surroundings It is considered that the concentration diffe

rences are maintained not only by passive physico chemical processes but also by metabolic reactions involving enzymic activity

It is known that sodium and chloride ions are found mainly in the interstitial or extracellular fluid and that potassium and phosphate are intracellular In recent years special attention has been paid to the problem of how this asymmetry of ionic distribution between the intra and extracellular phases arises and can be maintained (see further the introduction to part II)

A tissue may be divided into two morphologically different phases namely an extracellular and an intracellular phase The *intracellular* phase is considered to consist only of those cells specially concerned with the function of the tissue (e g for the muscle muscle cells) Assigned to the *extracellular phase* are the supporting connective tissue structures which surround the cells and certain fluids Certain cells are also usually included in the extracellular phase namely the fibroblasts in the connective tissue and the cells of the capillary walls

The extracellular phase thus consists of two parts the solid constituents (mainly proteins in connective tissue capillary walls and nerve fibres) and fluids (*interstitial fluid* blood lymph and the fluids in the connective tissue cells) The *interstitial fluid* which constitutes the *greatest part* of the extracellular volume consists of water derived from the plasma and contains ions and small molecules which also move outwards from the plasma i e the fluid resembles an ultrafiltrate

The extracellular volume (ECV) can be calculated if the total quantity and the extracellular concentration of a substance which is distributed in the entire extracellular space without entering or becoming attached to the cells is known No substance ideal for this purpose has as yet been found It is usual instead to determine approximate extracellular spaces by means of substances which are *mainly* distributed in the physiological extracellular space and to define the spaces found according to the substance used e g the sodium space mannitol space etc

Two groups of substances have been used in attempts at

determining the extracellular space 1 *Electrolytes* whose distribution resembles that of sodium or chloride (thiocyanate, iodide bromide sulphate and thiosulphate ions) 2 Small *non electrolytes* which pass through capillary walls but do not or only to a very small extent penetrate the cells (mannitol see PAGE 1962 sucrose see ROBERTSON and PEYSER 1956 raffinose and inulin see BOZLER 1959 1961)

Substances belonging to the first group are also distributed even if to only a small extent intracellularly and the ECV determined with these substances is therefore somewhat too large The ion most frequently used thiocyanate has the disadvantage that it binds to the plasma albumin (SCHEINBERG and KOWALSKI 1950) Thiosulphate has also been suggested since it does not bind to proteins (KOWALSKI and RUTSTEIN 1952) and radioactive sulphate has also been used (WALSER SELDIN and GROLLMAN 1953 1954)

As a rule the turnover of ions in the physiological extracellular space is rapid (for the sulphate and sodium ions the diffusion coefficient is about 60 % and 80 % of the diffusion coefficient in free solution respectively see JOHNSON 1955) Some of the non electrolytes belonging to group 2 are excreted rapidly and others are metabolised so that they also disappear from the plasma The distribution volume of inulin for example is often smaller than the corresponding chloride space

The majority of the studies on ion distribution have been made on striated muscle but several studies on heart tissue from different animals have also been made Heart tissue in mammals contains more water sodium and chloride and less potassium than the skeletal musculature which may indicate a larger extracellular space (MANERY 1954)

Since the literature on the electrolyte distribution in different tissues is very extensive reference may be made to review articles for more complete reference lists (MANERY 1954 BERGSTROM 1962 among others)

Relatively few studies have been devoted to comparisons of the electrolyte composition in different regions of the heart The aim of the present investigation was therefore to *attempt to elucidate the electrolyte distribution in the heart and to*

compare it with the distribution in skeletal muscle A further aim was to *attempt to determine the physiological extracellular volume and on the basis of the values obtained to calculate the intra and extracellular electrolyte concentrations*

Knowledge of these conditions appears to be necessary for the understanding of the nature of the spontaneous rhythmicity of the heart as this seems to depend on or at least linked to the ionic distribution and fluxes (see TLOBFLL 1962) It also seems important to study the electrolyte distribution in the heart in other states where the rhythmicity is altered The heart rate can be easily changed by vagal stimulation Studies were also therefore made *of the electrolyte distribution in the vagal stimulated heart and the exchange of ions between the heart and the blood on vagal stimulation in an attempt to find a basis for chronotropic changes* (see further the introduction to part II)

In these experiments the heart and skeletal muscle of toads were studied These animals have the advantage that the primary pacemaker is localized to a limited anatomical structure viz the sinus venosus and also that the atrium and ventricle are relatively homogeneous tissues

)

CHAPTER II

Methods

A. GENERAL METHODS

a) *Experimental animals*

Toads (*Bufo bufo* or in one series *Bufo viridis*) were used for the experiments. The animals were caught during May–August and were kept at 8–14°C. They were given no solid food but had access to water. The experiments were started in May and continued until February of the following year. Studies were made of different regions of the heart (sinus venosus, atrium and ventricle) and the skeletal muscle (m. sartorius) was also studied.

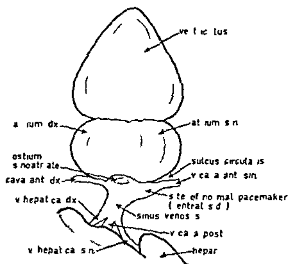


Figure 1. Toad heart (*Bufo bufo*), semi-schematic. The atria and ventricle have been displaced cranially in order to show the sinus venosus.

b) *The toad heart*

Fig. 1 shows a semi-schematic diagram of the toad heart which consists of the sinus venosus, the atrium divided into two chambers, and the ventricle. For further anatomical details reference may be made to ECKER-WIEDERSHEIM (1904).

1. *Structure of the toad heart*

For a microscopic description of the anatomy of the toad heart, see KISCH (1961). The *sinus venosus*, which is the normal site of origin of the electrical impulses in the toad heart, is characterized by a layer of striated muscle with the same histological properties as the musculature in the other regions of the heart. Between the single endothelial cell layer, which is considered to correspond to vascular endothelium (LINDNER 1957), and the muscle fibres, as also between the individual muscle fibres, are large quantities of longitudinal and transverse collagen fibres. The *atrium* has to a great extent the same construction as the sinus, but the muscle layer is considerably thicker and the connective tissue content smaller. The *muscle of the ventricle* has the same histological structure as the atrium and sinus.

There is no *specific conducting system* in the toad, as also in the other lower vertebrates, with the possible exception of certain turtle species (FANGE 1963). KISCH (1961), however, found profuse quantities of non-myelinated nerves in all regions of the heart.

KISCH (1961) never observed either *capillaries* or any white or red blood cells between the muscle fibres in any part of the heart, and this was considered to indicate that the toad heart lacks a coronary system (ECKER-WIEDERSHEIM 1904).

The extracellular space begins in the sub-endocardial region and is in communication with the spaces between the muscle fibres (LINDNER 1957).

Morphologically the borderline between the intracellular and the extracellular phase is difficult to define. In spite of the fact that the cell boundary is difficult to determine exactly, HUTTER and TRAUTWEIN (1956) stated that the heart muscle cells in the frog had a diameter of about 5 μ, and IWAMOTO

(1959) gave the same value for the fibres in the sinus venosus of the toad

SIMPSON and OERTELIS (1962) in electron microscopic studies on the ventricular musculature of the sheep heart found that the sarcolemma had invaginations which seemed to connect the intra and extracellular spaces SIIRO (1962) made also similar observations

An account of the normal morphology of *skeletal muscle* may be found in MAXIMOW & BLOOM (1942)

2 *The physiology of the toad heart*

Nutrition

Since the toad heart lacks a coronary system (ECKER WIEDERSHEIM 1904) the heart tissue receives its nutriment by means of the direct transport of solutes from the plasma in the heart lumen through the sponge like structure in the wall The sinus wall is only about 100 μ thick and the nutritional conditions are presumably quite adequate The wall thickness of the other heart regions is considerably greater

The localization of the pacemaker

IWAMOTO (1959) found that the primary pacemaker was localized to the ventral part of the orifice of the left anterior vena cava in the sinus venosus (see Fig 1) HUTTER and TRAUTWEIN (1956) maintained that the pacemaker was normally situated in the left region of the sinus venosus

c) *Dissection of the toads*

The toads were pithed after which the heart was dissected free from connective tissue and pericardium The sinus venosus atrium and ventricle were removed as also were skeletal muscle samples (sartorius muscle) The sinus venosus and also double samples of atrium ventricle and skeletal muscle were lightly blotted on filter paper and then weighed (see below) (Approximate weights of the preparations Sinus 5–15 mg atrium 15–35 mg ventricle 35–75 mg skeletal muscle 40–150 mg of wet tissue)

b) *The toad heart*

Fig. 1 shows a semi-schematic diagram of the toad heart which consists of the sinus venosus, the atrium divided into two chambers, and the ventricle. For further anatomical details reference may be made to ECKER-WIEDERSHEIM (1904).

1. *Structure of the toad heart*

For a microscopic description of the anatomy of the toad heart see KISCH (1961). The *sinus venosus*, which is the normal site of origin of the electrical impulses in the toad heart, is characterized by a layer of striated muscle with the same histological properties as the musculature in the other regions of the heart. Between the single endothelial cell layer, which is considered to correspond to vascular endothelium (LINDNER 1957), and the muscle fibres, as also between the individual muscle fibres, are large quantities of longitudinal and transverse collagen fibres. The *atrium* has to a great extent the same construction as the sinus, but the muscle layer is considerably thicker and the connective tissue content smaller. The *muscle of the ventricle* has the same histological structure as the atrium and sinus.

There is no *specific conducting system* in the toad, as also in the other lower vertebrates, with the possible exception of certain turtle species (FANGE 1963). KISCH (1961), however, found profuse quantities of non-myelinated nerves in all regions of the heart.

KISCH (1961) never observed either *capillaries* or any white or red blood cells between the muscle fibres in any part of the heart, and this was considered to indicate that the toad heart lacks a coronary system (ECKER-WIEDERSHEIM 1904).

The extracellular space begins in the sub-endocardial region and is in communication with the spaces between the muscle fibres (LINDNER 1957).

Morphologically the borderline between the intracellular and the extracellular phase is difficult to define. In spite of the fact that the cell boundary is difficult to determine exactly, HUTTER and TRAUTWEIN (1956) stated that the heart muscle cells in the frog had a diameter of about 5 μ, and IWAMOTO

The evaporation loss which is presumably exponential amounts during the first 6 minutes to about 6–7 % of the initial wet weight.

f) *Reference units for the concentrations of test solutes*

The amounts of the different test substances are given as concentration per unit wet weight of tissue. It is also possible to use fat free dry substance (DARROW, HARRISON and TAFFEL 1939) or non collagenous nitrogen as the reference units (LILIENTHAL *et al.* 1950).

Since data on the neutral fat content was not found in the literature this was investigated (for method see page 20 and results Table 1, page 21).

Since it was found that the water content varied very little between different toads (see chapter 7, table 11) and the neutral fat contributed to only a very small part of the wet weight (see Table 1, page 21) the wet weight seemed a valid reference unit.

B. CHEMICAL ANALYSIS

a) *Determination of sodium and potassium*

Sodium and potassium were determined by flame photometry. Heart and muscle tissue were first ashed by boiling in strong acid while plasma samples were merely diluted with water.

1. *Solutions*

Acid mixture. This consisted of 6 parts concentrated HNO_3, 4 parts concentrated $HClO_4$ and 1 part concentrated H_2SO_4 (all acids quartz distilled).

To prevent bumping quartz fragments were used. These had been boiled with sulphuric acid for two hours, washed with H_2O, boiled in HNO_3 for 30 mins and dried at 100°C.

Standard solutions of sodium and potassium were prepared by drying analysis grade sodium and potassium chlorides at 600°C for about 2 hours, after which the substances were cooled in a desiccator. The samples were weighed and dissolved in double distilled water.

d) *The collection of blood samples and the preparation of plasma samples*

Before the heart was dissected free blood samples were collected from the left aorta into heparinised capillary tubes the ends of which were then sealed in a flame and centrifuged at ≈ 9600 g for 3 mins The tubes were then cut open and the plasma samples were removed with calibrated constriction pipettes

e) *Weighing*

The tissue samples were freed from blood and tissue fluid by blotting on filter paper Duplicate samples of ventricle and skeletal muscle were enclosed in polyethylene tubes (weight of tube about 1 2 g) after which they were weighed on a balance accurate to 0 1 mg The plastic tubes prevented evaporation The sinus and atrium preparations were weighed repeatedly on an electro balance (Cahn Instrument Co Minnesota Ave Paramount Cal U S A max range 40 mg accuracy 30 μg precision 8 μg) after periods of different lengths after which the initial weight was determined by extrapolation In this way the error caused by the evaporation could be reduced The error was proportionally greatest for the sinus venosus preparation The weight curve is shown in Fig 2 where it may be seen that the evaporation is approximately proportional to time (at least the first six minutes)

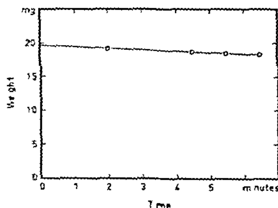


Figure 2 Weight curve of a tissue sample extrapolated to zero time

The evaporation loss which is presumably exponential amounts during the first 6 minutes to about 6–7 % of the initial wet weight

f) *Reference units for the concentrations of test solutes*

The amounts of the different test substances are given as concentration per unit wet weight of tissue It is also possible to use fat free dry substance (DARROW HARRISON and TAFFEL 1939) or non collagenous nitrogen as the reference units (LILIENTHAL *et al* 1950)

Since data on the neutral fat content was not found in the literature this was investigated (for method see page 20 and results Table I page 21)

Since it was found that the water content varied very little between different toads (see chapter 7 table 14) and the neutral fat contributed to only a very small part of the wet weight (see Table 1 page 21) the wet weight seemed a valid reference unit

B CHEMICAL ANALYSES

a) *Determination of sodium and potassium*

Sodium and potassium were determined by flame photometry Heart and muscle tissue were first ashed by boiling in strong acid while plasma samples were merely diluted with water

1 *Solutions*

Acid mixture This consisted of 6 parts concentrated HNO_3 4 parts concentrated $HClO_4$ and 1 part concentrated H_2SO_4 (all acids quartz distilled)

To prevent bumping quartz fragments were used These had been boiled with sulphuric acid for two hours washed with H_2O boiled in HNO_3 for 30 mins and dried at 100°C

Standard solutions of sodium and potassium were prepared by drying analysis grade sodium and potassium chlorides at 600°C for about 2 hours after which the substances were cooled in a desiccator The samples were weighed and dissolved in double distilled water

2 *Burning of tissue samples*

Sinus venosus preparations from two toads were combined. The atria from one toad consisted one sample. The ventricle and skeletal muscle samples were divided into two. Two modes of procedure were used in the burning process. In one of the methods the atrium and sinus preparations were placed in quartz crucibles (in order to obtain a more favourable ratio of sample/blank) and the ventricle and muscle preparations in pyrex glass beakers. 3 ml of acid mixture (the same amount for all samples) and two quartz fragments were added and the vessels covered with watch glasses were then heated electrically in a draught cupboard. After the burning the residue was dissolved in double distilled water (for sinus 5 ml, for atrial preparations 10 ml and for ventricular and skeletal muscle samples 25 ml).

In the other burning method the tissue samples were placed in 50 ml Kjeldahl flasks. 3 ml acid mixture and two quartz fragments were added and the flasks were then placed in a suction apparatus as described by NYDAHL (1949, 1950) (see Fig. 3) to safeguard against explosive combination of the perchloric acid vapour and organic material in the environment. After burning the residue was dissolved as previously.

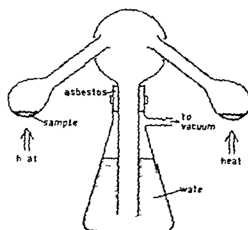


Figure 3. Apparatus for digestion with acid mixture as according to NYDAHL (1949, 1950). The seal between the suction flask and the neck of the upturned round flask consists of wet asbestos.

In both procedures blank samples were prepared in the same way as the tissue samples except for the presence of the tissue

Flame photometric analysis was performed as described below

3 *Preparation of plasma samples*

Duplicate samples of 25 μl plasma were measured with calibrated constriction pipettes and diluted with 5 ml double distilled water after which flame photometric determination was made as described below No surface active agent was added and neither were the plasma proteins precipitated prior to the analysis of sodium and potassium BERGSTRÖM and HULTMAN (1962) showed that the sodium content in human plasma before precipitation was about 1 5 % lower than after precipitation of plasma proteins In the toad where the plasma protein content is very low (see chapter IV) this interference can be regarded as negligible

4 *Flame photometric determinations*

The concentrations of sodium and potassium were determined with a flame photometer (flame photometer Eppendorf Netheler & Hinz GmbH Hamburg)—propane/air flame With this gas mixture no interferences between the sodium and potassium determinations were obtained Double distilled water was used as the zero reference

5 *Error of the method*

Six samples consisting of 0 2 ml human whole blood were burned after which the sodium and potassium contents were determined The coefficient of variation was ± 1 5 % for the sodium analyses and ± 2 % for the potassium analyses

b) *Determination of chloride*

The chloride content in the tissue and plasma samples was determined after burning The preparations were charred after the addition of an excess of calcium acetate so that a basic

ish should be obtained (thus preventing the release of hydrogen chloride during the heating process) Most of the resulting ash was dissolved in a water alcohol mixture The chloride content was then determined by mercurimetric titration

1 *Solutions*

Ethanol absolute 99 5 % (chloride free)

s diphenylcarbazone (Eastman Kodak 4459) 0 1 % solution in absolute ethanol

Brom cresol green (Merck AG Darmstadt Germany) 0 1 % in H_2O (stock solution)

Nitric acid 0 05 M

Mercuric nitrate 0 01 M

Sodium chloride 0 02 M

These solutions were prepared as according to DANIELSON (1963)

Calcium acetate solution 0 25 M

The calcium acetate solution was prepared by placing 50 g $CaCO_3$ according to Lawrence Smith in a 2 litre measuring flask and dredging the substance in about 800 ml distilled water after which 60 g distilled glacial acetid acid (approximately 65 ml) was added The calcium carbonate was dissolved by boiling and the solution was then cooled and 1 litre 95 % ethyl alcohol was added The solution was then made up to 2 litres with distilled water

In the preparation of the solutions double quartz distilled water was used in all cases solutes were analytical grade except when stated

2 *Preparation of samples*

The heart and muscle preparations were placed in small quartz crucibles (diameter 14 mm height 25 mm) and 0 3–0 5 ml 0 25 M $Ca(Ac)_2$ solution was added The samples were dried under a heat lamp to prevent boiling during the charring process which was done in an oven for 12–15 hours at 560°C The ash was dissolved in a mixture of water and alcohol as described below

1 ml distilled water was added to the sample in the quartz crucible and the ash was stirred with a glass rod, after which the solution and deposit were poured into a centrifuge tube. The remaining ash was stirred further in 1 ml distilled water and poured into the centrifuge tube. In order to transfer the deposit quantitatively into the centrifuge tube the crucible was washed out with absolute alcohol (5–6 ml).

The sample volume was adjusted to 12 ml with alcohol and the sample was then centrifuged. The supernatant solution was poured into a 25 ml beaker. The deposit was washed once with a water-alcohol mixture (1 ml H_2O and 6 ml alcohol) and the washing fluid was added to the remaining sample.

The plasma samples were prepared in the same way as the others. 50 μl were burned with 0.3–0.5 ml calcium acetate solution to obviate the influence of the proteins on the final determination.

3. *Titration of chloride*

Chloride was determined by means of mercurimetric titration in a mixture of ethanol and water as solvent at a pH of 3.5 with photometric estimation of the end point (DANIELSON 1963).

4. *Error of the method*

10 samples (weight of each sample about 75 mg) from the same rabbit muscle, cut into small pieces, were burned with calcium acetate solution, after which the samples were treated as above. The coefficient of variation was ± 5 %. One reason for the large variation coefficient may be that the actual chloride content may vary within the same muscle (cf. chapter IV, page 37). In a series of 5 identical plasma samples of 50 μl each the coefficient of variation was ± 3 %.

c) *Determination of proteins in the plasma*

The plasma proteins were determined by the biuret reaction as according to KNIGHTS, MACDONALD and PLOOMPUU (1962). The biuret reagent gives a blue colour with proteins. The

colour of the sample solution was compared with that of a protein solution with a known content which was treated in the same way.

1. *Solutions*

The chemicals used were of analytical grade.

Concentrated carbonate free sodium hydroxide was prepared as according to KOLTHOFF and SANDELL (1961).

Sodium hydroxide 10 % was prepared from the above solution.

Biuret solution. 1.5 g copper sulphate ($CuSO_4$ 5 H_2O) and 6.0 g potassium sodium tartrate were dissolved in 500 ml distilled water in a measuring flask. 300 ml 10 % sodium hydroxide were added during stirring and the solution was then diluted to 1000 ml with distilled water.

Albumin solution 4 %. This solution used as a standard solution was obtained by diluting 20 % albumin solution (Kabi, Stockholm, Sweden) with distilled water.

2. *Procedure*

To three tubes containing 50 µl plasma, 50 µl distilled water and 50 µl of a standard human albumin solution (4 %) respectively were added 2.0 ml biuret solution. The tubes were then shaken and allowed to stand for 30 minutes, after which the absorbance at 540 nm was measured with distilled water as blank. The protein concentration in the sample was calculated from a calibration curve.

3. *Error of the method*

The plasma protein concentration in five samples containing 50 µl of the same plasma was determined as described above. The coefficient of variation was ± 1 %.

d) *Determination of water and neutral fat contents*

The tissue preparations were dissected out, blotted dry and weighed repeatedly and then dried on small pieces of aluminium foil in an oven at about 90–100°C for at least

2 hours, often 10–12 hours, then re-weighed. The water content was obtained by subtraction of the dry weight from the wet weight at zero time. BERGSTRÖM (1962) reported that drying for 2 hours was sufficient to obtain a constant weight.

Both the water and neutral fat content were determined in certain preparations. The preparations were fixed on to small wire hooks (weight about 10 mg), dried at 90°C for about 10 hours and re-weighed. They were then placed in 10 ml petroleum ether (Baker Analyzed Reagent, boiling point 30–60°C) for 2–3 hours, dried at 90°C for about 30 mins and weighed again. The fat content was determined as the difference between the dry weight and the weight after extraction with petroleum ether. The extraction time should have been sufficient as FLEAR and FLORENCE (1961) found complete extraction of fat after 10 mins in samples weighing 9–16 mg (wet tissue). For the results of the neutral fat determinations, see Table 1.

Table 1

The neutral fat content of the heart and skeletal muscle. The table shows the mean values ± one standard deviation obtained from the analysis of preparations from 10 toads (only 7 determinations on the sinus venosus)

Preparation	g neutral fat/100 g wet tissue
Sinus venosus	0.7 ± 0.6
Atrium	[illegible]0 ± 1.4
Ventricle	1.1 ± 0.[illegible]
Sartorius muscle	0.5 ± 0.3

C. RADIOACTIVE ANALYSES

a) *Gamma spectrometry (analysis of Na^{22}, Na^{24}, K^{42}, Br^{82} and I^{131})*

1. *Apparatus and preparation of samples*

The gamma spectrometer had a 1¾" NaI(Tl) well type crystal combined with a photomultiplier (type RCA 6655), the amplification of which did not change with the impulse frequency (ÖBRINK and ULFENDAHL 1959). The high voltage of the photomultiplier was obtained from aggregates with very good stability (Tracerlab Precision High Voltage Supply, long period stability 0.02 %/24 hours). The supply voltage of the apparatus was also stabilized (228 V ± 0.2 %). The linear

amplifier (Tracerlab RLA 1) had good non over load properties Two pulse height analysers (Tracerlab RLA 5 S) were used

The gamma energy spectra were recorded as according to ÖBRINK and ULFENDAHL (1959)

Two types of analysis tubes of polythene plastic were used *The larger type (16 × 59 mm volume 6 5 ml) fitted exactly* into the well of the crystal (Used in the analysis of Na^{24} K^{42} Br^{82} singly and in the analysis of mixtures of Na^{24} and Br^{82} and of Br^{82} and I^{131}) The samples were inserted directly into the tubes in such a way that comparable conditions of analysis were present for all samples

The samples were placed in small plastic tubes (outer measurement 8 × 38 mm volume 1 ml) The sample volume never exceeded that which gives 98–100 % relative counting activity In the analysis of the Na^{24} and K^{42} mixture the beta radiation was *screened off especially that of K^{42} Each plastic tube* was placed in a brass cylinder (2000 mg/cm² which gives small Bremsstrahlung because of low atomic number 28 − 29) in order to obtain absorption of all beta particles with energies below 4 0 MeV The brass cylinder containing the sample tube was placed in a larger plastic tube

2 *Error of measurement*

A Cs^{137} sample was counted 50 times (energy channel = 0 004 MeV set on the steep part of the 0 66 MeV peak) and a coefficient of variation of ± 0 25 % was obtained i e the theoretical counting error (DANIELSON and SJÖSTRAND 1964) This showed the good stability of the apparatus

In all cases the necessary corrections for radioactive decay were made

3 *Determination of individual isotopes (Na^{24} K^{42} and Br^{82})*

In the analyses of individual radioactive isotopes broad energy channels (wide open windows) were used and it may be assumed that the error in the analysis of one isotope was approximately $\pm\sqrt{n}$ where n is the number of impulses recorded (see SCHMEISER 1961) In the analysis of Na^{24} or Br^{82}

all impulses below 1.50 MeV were recorded and in the analysis of K^{42} or Na^{24} all impulses below 3.5 MeV. As a rule at least 10 000 impulses from each sample were counted.

4. *Determination of radioactive isotopes in mixtures*

The determination of isotopes in mixtures (Br^{82} and I^{131}, Na^{24} and Br^{82} or Na^{24} and K^{42}) was carried out according to the method of ÖBRINK and ULFENDAHL (1959). In order to reduce the error broad energy channels were used, thus giving a higher counting rate. By selecting suitable energy values for the channel boundaries the effects of the instability of the apparatus can be reduced (cf. DANIELSON and SJÖSTRAND 1961). In the analysis of Br^{82} and I^{131} the channel boundaries were chosen such that in channel I all energies below 1.50 MeV were recorded and in channel II energies between 0.40 and 1.50 MeV. In the analysis of Na^{24} and Br^{82} all energies below 1.0 MeV were recorded in channel I and energies between 1.0 and 4.0 MeV in channel II. In the analysis of a mixture of Na^{24} and K^{42} energies between 1.1 and 2.1 MeV were recorded in channel I and energies between 1.1 and 3.5 MeV in channel II. By frequent counting of the standard samples (approximately every fifth sample was a standard) any drift in the channels could be corrected for.

At least 5000 impulses from each isotope were recorded in all samples. The statistical counting error was never greater than ± 1.4 % and the total error in the determination of isotopes in mixture probably did not exceed ± 3 % in any analysis.

b) *Liquid scintillation counting (analysis of C^{14} and S^{35})*

For the analysis of C^{14} and S^{35} a method described by KALBERER and RUTSCHMANN (1961) was used based on the oxygen combustion method reintroduced by SCHÖNIGER (1955).

Preparations containing C^{14} and S^{35} are burned in pure oxygen. The products (carbon dioxide, sulphur dioxide and sulphur trioxide) are then absorbed in a liquid mixture which is later mixed with toluene containing phosphors for liquid scintillation counting.

1 *Solutions*

The *absorption solution* in the C^{14} and S^{35} determinations was 15 ml of a mixture of 120 ml distilled 2 amino ethanol diluted to 1000 ml with analytical grade methanol The volume used can quantitatively absorb the combustion products from 200 mg substance (including the filter paper) The *scintillator solution* consisted of toluene (Baker s toluene Analyzed Reagent) to which was added 4 0 g PPO (2 5 diphenyl oxazol) and 100 mg POPOP (1 4 di [2(5 phenyloxazolyl)] benzene) to 1000 ml toluol

2 *Preparation of samples*

For combustion 1 litre pyrex round flasks with tightly fitting glass stoppers (NS 29 or 24) were used The appearance of the flask and stopper is shown in Fig 4 A platinum wire was

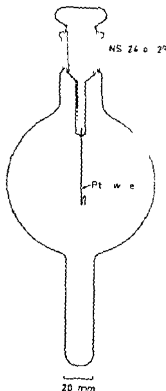


Figure 4 1 litre burning flask with glass stopper into which a Pt wire is fused

fused on to the inner side of the stopper A blind glass tube (length 90 mm inner diameter 20 mm) was fused on to the bottom of the flask to contain the absorption solution during the burning process (One litre flasks suitable for burning preparations up to 0–250 mg in weight) Optimal combustion conditions are obtained if only about a third of the oxygen content of the flask is used The preparations were placed on special burning papers (see Fig 3) i e quantitative filter papers (No 589 Schleicher and Schuell AG Feldmeilen Zurich Switzerland) weighing about 130 mg each (The ash content in the filter paper was approximately 0 01 %) A piece of platinum net (1 5 × 1 5 cm 48 mesh perforated Pt plate) was placed around the preparation after which the preparation was fixed on to the platinum wire in such a way that the net was open at the top and sides

3 *Procedure*

Combustion was carried out as follows

The flask with the absorption solution was lowered into a thermos vessel with a mixture of dry ice and acetone for 3 minutes which sufficed to cool the absorption solution to – 60 C Oxygen was then blown into the flask for 1 minute The contact surface of the stopper with the preparation to be burned was damped with water to obtain an effective gas tight

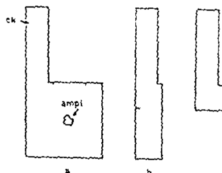


Figure 3 Combustion paper with sample (a) is folded along the dotted lines (a and b) to form a small packet which is enclosed in a piece of Pt net and attached to the Pt wire on the glass stopper (see Fig 4)

lock a corner of the filter paper was lit and the stopper was lowered quickly into the oxygen filled flask After burning the flask was left in the cooling mixture for two minutes to allow the gas and platinum wire to cool and the flask was then rotated mechanically for 20 minutes

10 ml scintillator solution were pipetted into the counting flasks to which 10 ml of the absorption solution were added and the samples were then analysed

4 *Apparatus*

A Packard Tri Carb Liquid Scintillation Spectrometer model 314 EX 2 (Packard Instrument Company LaGrange Ill U S A) was used at + 3°C The voltage of the photomultiplier was 1150

The stability of the spectrometer was tested as follows One S^{3} sample was counted 62 times during a period of about 10 hours The coefficient of variation was ± 0 78 % The theoretical statistical counting error for the number of impulses counted was ± 0 67 % i e the stability of the spectrometer was good

5 *Measuring conditions*

The channel setting is shown in Fig 6 The background was of the order of size of 75 cpm in the large energy channel The samples were as a rule counted 3 × 10 minutes each at least 10 000 impulses usually being recorded in the wider channel each time

The spectral curve for S^{3} was determined The exact energy values were not determined only the appearance of the spectral curves being studied (The abscissal values in Fig 6 are therefore only approximate) Since S and C^{14} have almost the same energies (0 167 and 0 155 MeV respectively) only the spectral curve for S was determined

The quotient between the net counts on the two energy channels gives an idea of any variations in quenching from sample to sample i e quenching causes a shift of the whole

spectral curve towards lower energies and some extinction of impulses The coefficient of variation for the quotient between the two energy channels was ± 1 5 % The reason for these variations in the quotient may be 1 Quenching variations 2 Instability in the electronic apparatus (see page 26)

Lack of differences in quenching is probably due to the large volume of quenching substances such as methanol and ethanolamine which mask the effects of small variations in the sample quenchers e g water and ash particles The main reason for the small variations in the quotient between the two energy channels was probably instability of the electronic apparatus

6 *Error of the method*

To determine the error of the method 10 µl S solution were pipetted on to five combustion papers dried burned and analysed The coefficient of variation was ± 2 % (The statistical counting error for the same number of impulses was ± 0 5 %)

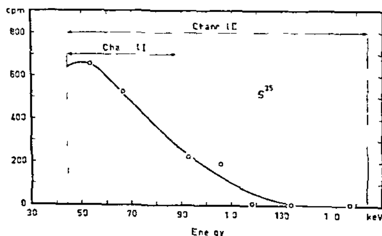


Figure 6 The channel settings used in the analysis of C^{14} and S^{35} by liquid scintillation counting The abscissal energy values are only approximate

7 The presence of carriers

Good reproducibility in the analysis of C^{14} is obtained because adsorption to glass walls, platinum net and wires is avoided, since relatively large quantities of carbon dioxide and water arise from inactive material in the burning process.

In the combustion of sulphur containing substances sulphur dioxide and sulphur trioxide are formed. Addition of inactive sulphates resulted in very varying recoveries of the radioactive isotope. This may be because the sulphur oxides formed do not react chemically with the absorption solution but are only dissolved in the small amount of water formed during combustion. If much sulphur is present the amount of water formed may be insufficient to dissolve all the sulphur oxides formed. Therefore in the analysis of S^{35} in the tissue samples no inactive sulphur compound was used as a carrier.

In order to investigate whether there was any adsorption of S^{35} to the platinum wire and platinum net after the burning of a S^{35} sulphate sample, these were placed in absorption solution for 1–2 hours, after which the whole absorption solution was analysed for S^{35}. No difference was found between these samples and ordinary background samples.

D SOLUTIONS

Normal Ringer solution: 6.5 g NaCl (111.2 mM), 0.2 g KCl (2.68 mM), 0.2 g $CaCl_2$ (1.80 mM), 0.1 g $NaHCO_3$ (1.19 mM) and 1 g glucose (5.6 mM) per litre H_2O (pH = 7.2).

Radioactive Ringer solutions were obtained by the addition of $Na^{24}Cl$, $Na^{22}Cl$, $K^{42}Cl$, NH_4Br^{82}, NaI^{131} or $Na_2S^{35}O_4$ to the normal solution. Na^{24} and K^{42} were obtained as $NaHCO_3$ and K_2CO_3, which were converted to chlorides with HCl.[1] Br^{82} was obtained by neutron radiation of NH_4Br and was dissolved in distilled water.[1] S^{35} was obtained by a (n, p) reaction in the form of carrier free $Na_2S^{35}O_4$ in aqueous solution.[2]

[1] These isotopes were obtained from AB Atomenergi, Stockholm, Sweden.

[2] Na^{22}, S^{35} and C^{14} labelled carbohydrates were obtained from The Radiochemical Centre, Amersham, England.

In studies of the distribution of C^{14} labelled carbohydrate molecules C^{14} was added to Ringer solution in the form of C^{14} mannitol $(OHCH\,(CHOH)_4\,C^{14}H\,OH)$, C^{14} sucrose (L) or C^{14} maltotriose (U). The two latter substances were uniformly labelled. No check of the radiochemical purity was made immediately prior to use.

All radioactive material was added to the normal Ringer solution.

The quantity of radioactive material injected into the animals is given when the experimental data are presented.

CHAPTER III

Total sodium and potassium content in the cardiac and skeletal muscle

The distribution of sodium and potassium in different tissues has long been the object of great interest. Since these ions are considered to be intimately associated with impulse formation and propagation in all excitable tissue (see further introduction to part II) interest has also been centred on their distribution in the heart. DAVIES *et al* (1952) found in the ox heart a very high sodium concentration in the conduction system, a lower value in the atrium and the lowest in the ventricle, while the distribution of potassium was the reverse of that of sodium. MAZEL and HOLLAND (1958) reported similar findings in the frog heart.

The aim of the experiments reported here was to determine the distribution of electrolytes between the intra- and extracellular spaces in different regions of the heart and to compare this with the electrolyte distribution in skeletal muscle.

It was first necessary to determine the ECV and the total content of sodium and potassium in the different parts of the heart, in skeletal muscle and in the plasma. The sodium and potassium contents in biological tissues can be determined in several ways, e. g. by analysis of the stable isotopes or with the aid of radioactive isotopes. One difficulty with regard to the use of radioactive potassium isotopes in the determination of the total potassium in cardiac and skeletal muscle tissue is that potassium requires a very long equilibration time, since it is mainly intracellular and much of this exchanges very slowly with the extracellular potassium (WEATHERALL 1962 *a*). WEATHERALL (1962 *b*) found that in the rabbit atrium approximately 20 % of the potassium is exchanged very slowly

and that the major part of this was localized to the sarcosomes Since only short lived radioactive potassium isotopes are available these decay to too low activities before complete equilibration has been attained and recourse must be had therefore to stable potassium Analysis of stable sodium was also made

METHODS

Toads were used for the experiments these being kept as described in chapter II (With regard to the significance of the periods of captivity see below)

The dissection of the toads the collection of blood and other tissue samples combustion and other sample preparations and the final determination by flame photometry were performed as described in chapter II

RESULTS

The results have been summarized in the accompanying tables Table 2 summarizes the determinations of sodium and potassium in the plasma No definite difference in values was found between toads which had been kept in captivity for different lengths of time and the table shows only the combined results Table 3 shows the sodium and potassium content in the heart and skeletal muscle of toads after different periods of captivity It may be seen that the sodium content in the different tissue preparations remains on the whole constant except for the skeletal muscle where the sodium content increases significantly after long periods of captivity ($P < 0\ 01$ on comparison of newly captured toads with toads captured 6–8 months previously) The potassium content is significantly lower ($P < 0\ 01$) in all tissue preparations after 6–8 months captivity than in newly captured toads

Table 2

Sodium and potassium concentrations in toad plasma (*Bufo bufo*) regardless of period of captivity The tables show the mean values ± one standard deviation the variation range and the number of determinations

	meqv/l	Variation range	No of determinations
Sodium	1 2 ± 4 8	110 – 1 33	50
Potassium	3 1 ± 0 6	1 8 – 4 5	40

Table 3

The sodium and potassium concentrations in the cardiac and skeletal muscle after varying periods of captivity. The table shows the mean value, standard deviation and number of determinations.

Preparation	Sodium Meqs/kg wet tissue	Sodium No. of determination	Potassium Meqs/kg wet tissue	Potassium No. of determination
Toads in captivity < 3 months				
Sinus venosus	77.4 ± 10.3	14	24.[illegible] ± 2.0	14
Atrium	[illegible]2.3 ± [illegible].8	14	3[illegible] ± [illegible].4	14
Ventricle	3[illegible].9 ± [illegible]	14	[illegible] ± [illegible].8	14
Skeletal muscle	24.2 ± 3.[illegible]	[illegible]0	[illegible]3 ± 3.[illegible]	[illegible]0
Toads in captivity approx. 6 months				
Sinus venosus	81.0 ± 10.[illegible]		20.[illegible] ± 1.[illegible]	4
Atrium	63.7 ± 8.[illegible]	8	2[illegible].9 ± [illegible]6	8
Ventricle	3[illegible].0 ± 4.4	10	3[illegible].2 ± [illegible].1	10
Skeletal muscle	[illegible]6.0 ± [illegible]	[illegible]	[illegible]1 ± [illegible]	10
Toads in captivity approx. 8 months				
Sinus venosus	67.1	3	13.9	3
Atrium	6[illegible].5 ± 14.9	[illegible]	1[illegible] ± [illegible]3	6
Ventricle	33.8 ± [illegible]	[illegible]	34.0 ± 6.4	6
Skeletal muscle	41.0 ± [illegible]	6	43.8 ± 2.1	[illegible]

Table 4

The ratio of Na/K in different heart regions after varying periods of captivity.

Preparation	Mean value	Variation range	No. of determination
Toads in captivity <3 months			
Sinus venosus	3.[illegible]	[illegible]9 – 3.[illegible]	14
Atrium	1.[illegible]	1.6 – [illegible]0	14
Ventricle	0.[illegible]	0 – 0.[illegible]	14
Skeletal muscle	0.3	0.3 – [illegible]	20
Toads in captivity approx. 6 months			
Sinus venosus	3.8	3.[illegible] – 4.[illegible]	4
Atrium	[illegible].8	1.8 – 4.[illegible]	8
Ventricle	1.0	0.8 – 1	10
Skeletal muscle	[illegible]	0.3 – 0.[illegible]	9
Toads in captivity approx. 8 months			
Sinus venosus	4.9	4 – [illegible]	3
Atrium	3.[illegible]	2.1 – 6.8	6
Ventricle	1.2	0.[illegible] – 2.0	6
Skeletal muscle	0.9	0.8 – 1.1	6

Table 4 gives the Na/K ratios (of molar concentrations) in the different tissue preparations after varying periods of captivity. For all tissue preparations the ratio increases after a long period of captivity this being due mainly to a decreased potassium content.

DISCUSSION

The results obtained on the sodium and potassium content in the atrium and ventricle conform well with earlier findings (JOJIMA and KURIYAMA 1957). The electrolyte content in the tissues of frogs and toads varies greatly with the time of year and the length of time which the animals have been kept in captivity (PORSZASZ *et al.* 1962). As may be seen in Table 3 the tissue potassium in particular alters. A possible explanation for this change may be variations in the general condition of the animals or their metabolism during captivity. The temperature at which they are kept (8–14°C) may also be of some significance to the electrolyte composition in the tissues. It is known that tissues *in vitro* that are subjected to low temperatures lose potassium (HASHISH 1958, ELIASSEN and LEIVESTAD 1961). In this investigation however no investigation of the body temperature of the toads in relation to the ion concentrations was made.

Possible explanations for the variations in the electrolyte composition of the tissues

Since sodium is mainly extracellular a tissue with a large extracellular space should have a high sodium content.

The sinus venosus contains more sodium per unit weight than the atrium and ventricle. This may be an expression of the fact that the sinus venosus contains more connective tissue than the atrium and ventricle since connective tissue is known to contain more sodium than muscle tissue (see DAVIES *et al.* 1952, KISCH 1961 *inter alia*). A second possible explanation may be that the sinus venosus and the conduction system contain a larger quantity of extracellular water than the atrium and ventricle. A third possibility is that the conduction system has

a different intracellular ion composition than other tissues e g atrial muscle, and that there is a continual transition between the ionic compositions of the pacemaker conduction system and that of the skeletal muscle The latter tissue has no pacemaker properties and furthermore has a completely different ionic composition from the sinus venosus The atrium and ventricle are latent pacemakers These tissues have an ionic composition that lies midway between that of the conduction system and that of the skeletal muscle

To assess the last alternative the intracellular sodium and potassium concentrations had to be determined in the different tissues These results are reported in chapter VII

CHAPTER IV

The chloride content and the halide spaces in the cardiac and skeletal muscle

The distribution of the anions in the tissues has not received as much attention as that of the cations since it has been considered that anions are passively distributed and do not thus contribute to the resting potential to any noteworthy degree One difficulty with regard to chloride ions in different tissues has been that of finding a satisfactory method of analysis These factors are in part at least responsible for the paucity of information regarding the distribution of the chloride ion The data in the literature on the intra and extracellular chloride contents and chloride distribution in cardiac and skeletal muscle tissue vary greatly from author to author depending on which method has been used in analysing the chloride and which substance has been used for the determination of the extracellular volume

The analysis of chloride in biological material especially in tissues which has been a difficult problem comprises two principal operations 1 The preparation of a solution which contains *all* chloride in the tissue sample but no substances which interfere with the final determination 2 The measurement of chloride in the solution by means of a colorimetric titrimetric or other method

The most commonly used preparation method has been wet charring with concentrated nitric acid (VAN SLYKE 1923) followed by Volhard titration (see for example KOLTHOFF and SANDELL 1961) Since this wet charring method has given doubtful or apparently erroneous results it has been modified repeatedly The modifications have been compared directly with other methods on series of tissue samples but the results have varied greatly For example chloride values obtained by

a dry charring method were the same as those obtained with VAN SLYKE's wet charring method (1923) in one comparison (CASTER 1955) but were higher in another (CHITE and WEST 1955). Methods in which extracts of fresh muscle tissue with warm water or diluted sodium nitrate solution were used gave chloride values which were about 60 % higher than the values obtained by VAN SLYKE's method (HEILBRUNN and HAMILTON 1942).

The disparities in the chloride results obtained by different methods have been greatest in analyses of liver and muscle which have a low content of chloride in relation to protein and other organic substances. As a rule it has been assumed that the method which gave the higher results was the more correct one, and the lower results were considered to be due *inter alia* to inadequate extraction of chloride or loss during the wet or dry charring process (COTLOVE 1963 *a*).

Some of the methods available for chloride determination in biological material have fulfilled the ordinary demands of accuracy, i. e. reproducibility, complete recovery of the chloride added and agreement of the results with those of another method. These criteria do not preclude errors, however. COTLOVE (1963 *a*) got round the difficulties introduced by possible incomplete chemical recovery by making use of the isotope dilution principle. A known quantity of Cl^{36} with a known specific activity was mixed with the biological sample. This was burned and it was assumed that there was complete mixing between stable and radioactive chloride. The true chloride content in the biological sample could then be calculated by determining the reduction in the specific activity of the tracer due to dilution with the stable chloride in the sample. In isotope dilution analysis it is of course necessary that the final chemical determination method is accurate. COTLOVE (1963 *b*), using the isotope dilution method as a reference, compared other chemical analyses of chloride in tissue.

The values which COTLOVE (1963 *a*, *b*) found for the chloride content in the muscle and liver were lower than most of the previous results. Some of the values reported in the literature differed by no more than 20 % from those obtained by the isotope dilution method, while others deviated much more.

For the analysis of biological fluids direct dilution and titration were found to be accurate procedures

The variation in the chloride content in many tissue samples can occur through differences in the condition of the animals the site from which the sample is taken and varying contents of such tissue components as blood interstitial fluid and connective tissue While such variations may explain differences of up to ± 20% (COTLOVE 1963*a*) in comparisons between tissue samples from different animals the larger variations must be ascribed to errors in the analytical methods

As a rule the intracellular concentrations of chloride are lower than the extracellular Several different theories have been advanced to explain this asymmetric chloride distribution in muscle tissue According to one theory (FENN 1936 HASTINGS and EICHELBERGER 1937) the muscle membrane *in vivo* is completely impermeable to chloride ions It has been shown under *in vitro* conditions however that the muscle membrane is permeable to chloride ions (BOYLE and CONWAY 1941 ADRIAN 1960 1961 HARRIS 1960)

According to another theory (BOYLE and CONWAY 1941 and others) the muscle membrane *in vivo* is *slightly* permeable to chloride ions which are distributed completely passively under the electrochemical forces i e there is a Donnan distribution According to this concept the ratio for the extra and intracellular chloride concentrations $[Cl]_e/[Cl]_i$ is determined by the resting potential which is normally considered to be the greater part of a diffusion potential for potassium ions

A third theory (YANNET and DARROW 1940 HEILBRUNN and HAMILTON 1942 SHANES 1950) suggests that there is some amount of nondiffusible (very slowly exchanging) or bound chloride which is localized intra or extracellularly According to SIMON *et al* (1957) TROSHIN (1958) and LING (1960) a certain degree of intracellular binding of monovalent ions occurs but not definitely of chloride ions only These hypotheses have not received strong experimental support however

KEYNES (1963) found in squid axons that the chloride distribution was not in accordance with that expected from a passive distribution between the axoplasm and the extracellular fluid The determination of the activity coefficient in the axoplasm

showed that this was almost the same as for chloride in free solution which appears to preclude the concept that the chloride is bound inside the axon to any great extent

Some investigations have also been made on the chloride distribution in the heart LAMB (1961) showed that the intra cellular concentration of chloride in rat atria was 19 5 meqs/kg water while the plasma chloride concentration was 108 meqs/kg The equilibrium potential calculated by putting these values into the Nernst equation was 47 mV

The chloride content in different regions of the heart is still very uncertain Furthermore the data on the intracellular chloride concentration vary considerably The aim of the present investigation was therefore to determine by means of a method which seemed reliable the chloride content in different regions of the heart and in skeletal muscle and to calculate the chloride spaces A further aim was to compare the distribution of different halide ions in the heart This is of some interest since for example bromide ions are often used instead of chloride ions in distribution and ionic flow studies in tissue preparations and in ECV determinations

METHODS

In a series of 10 toads the chloride content was determined in different regions of the heart in skeletal muscle and also in plasma The experiments were performed on newly caught toads The dissection of the toads the collection and preparation of samples and the determinations were performed as described in chapter II

Determination of Br^{8} bromide and I^{131} iodide spaces in the heart and skeletal muscle

In order to obtain an idea of the bromide and iodide spaces in different regions of the heart and in skeletal muscle approximately 1 ml radioactive Ringer solution containing about 10 μC Br^{8} (specific activity 74 mC/g Br) and 10 μC I^{131} (carrier free) was injected into the posterior lymph sac of nine toads after which these radioactive isotopes were allowed to distribute for about 30 hours Tissue and blood samples were

then taken as described in chapter II The plasma samples were transferred quantitatively to analysis tubes as also were the heart and muscle preparations and analysed for their content of Br^{8} and I^{131} by means of gammaspectrometry (see chapter II page 21)

Determination of proteins in plasma

The plasma proteins were determined in a series of 10 toads as described in chapter II page 19

RESULTS

The chloride content in heart and muscle tissue

The results of the chloride determinations in heart and skeletal muscle in toads (*Bufo bufo*) are shown in Table 5 It may be seen in the table that the content of chloride in the sinus venosus is greatest followed by the atrium and then the ventricle The heart as a whole has a higher content than skeletal muscle

The chloride content in plasma

The mean chloride concentration in the plasma from 10 toads was 91 3 ± 5 5 meqv/litre (± 1 S D)

The halide spaces in the heart and skeletal muscle

The volume of water in the extracellular space in a sample can be calculated if the total quantity and concentration of a substance which is known not to penetrate the cell membrane

Table 5

The chloride content in the heart and skeletal muscle of newly caught toads (*Bufo bufo*) The table shows the mean values ± one standard deviation

Preparation	meqv Cl^- per kg wet tissue	No of determinations
Sinus venosus	2 0 ± [illegible]	5
Atrium	47 2 ± 4 6	10
Ventricle	23 8 ± 1 4	9
Sartorius muscle	15 0 ± 1 5	10

is determined Several substances have been used for this purpose It has been assumed here provisionally that chloride bromide and iodide ions are distributed only in the extra cellular space This is not entirely correct since a certain fraction of these ions do enter the cells but a calculation based on the assumption that they are entirely extracellular give some idea of the extracellular space and may be of some guidance in comparisons of the size of this space in different heart regions and in skeletal muscle

Calculation of the results

The calculation of the different spaces refers to 100 g wet tissue The following notation will be used

$[H_2O]_p$ = ml water per litre plasma
$[Cl]_p$ = chloride concentration in plasma (meqv/l)
$[Br^{82}]_p$ = Br^{8} concentration in plasma (cpm/l)
$[Cl]_e$ = chloride concentration in the extracellular water (meqv/l)
$[Br^{82}]$ = Br^{8} concentration in the extracellular water (cpm/l)
$[Cl]_m$ = meqv chloride per 100 g wet tissue
$[Br^{82}]_m$ = cpm Br^{8} per 100 g wet tissue

The plasma water was not determined but was calculated on the basis of the protein concentration according to the equation below given by EISENMAN MACKENZIE and PETERS (1936) and tested for protein concentrations from about 3 to 8 g/100 ml plasma

$$[H_2O]_p = 984 - 7\ 18 \text{ protein concentration (in g/100 ml plasma)}$$

The mean protein concentration in plasma from 10 toads was 2 6 ± 0 5 g/100 ml plasma (duplicate samples from each toad) which gives a water concentration of 965 g/l plasma The protein concentration in the toad plasma is low compared with *the mean value of 7 5 g/100 ml plasma in man* (BERGSTRÖM 1962) In comparison it may be mentioned that rats have a plasma protein concentration of 3 5 g/100 ml plasma (RAUEN 1956)

On calculating the chloride concentration in the extracellular water consideration should perhaps be taken of the probable

existence of a Donnan equilibrium for the diffusible ions between plasma and the extracellular space. Since plasma contains proteins which, it is assumed, do not enter the extracellular space, the concentration of chloride in the extracellular water should be higher than in the plasma. In calculations in man a Donnan ratio (r) of 0.96 has been used (see VAN SLYKE 1923 and BERGSTRÖM 1962). In calculations on the toad the Donnan factor will be less important since the plasma protein content is so low. The permeability conditions in toads may be different from those in mammals and since neither the plasma pH, the isoelectric points of the plasma proteins nor the protein fractions themselves were investigated it is difficult to correct for a Donnan distribution.

If a Donnan ratio is used, the following equation holds:

$$[Cl] = \frac{[Cl]_p \cdot 1000}{r \cdot [H_2O]_p}$$

If no Donnan factor is used r will be put equal 1.

The extracellular Br^{82} and I^{131} concentrations were calculated in the same way. In doing this it was presupposed that Br^{82} and I^{131} were distributed similarly to chloride ions with the same Donnan ratio.

In the calculations correction was made for the solid content of plasma.

The chloride space was calculated according to the following formula:

$$\text{The chloride space} = \frac{[Cl]_m \cdot 100}{[Cl]} \text{ ml/100 g wet tissue}$$

The Br^{82} bromide and I^{131} iodide spaces were calculated similarly.

The chloride, bromide and iodide spaces in different tissues

In these calculations no Donnan correction was used since this factor is difficult to determine. The mean values for chloride space determinations in toads (*Bufo bufo*) are shown in Table 6. The preparations consisted of sinus venosus, atrium, ventricle and skeletal muscle (sartorius).

Table 6

The chloride spaces in the heart and skeletal muscle of the toad (*Bufo bufo*). The table shows the mean values ± one standard deviation

Preparation	ml per 100 g wet tissue	No. of determinations
Sinus venosus	[illegible]6.0 ± [illegible]	
Atrium	50.3 ± 8	10
Ventricle	2[illegible] 4 ± 2.1	[illegible]
Sartorius muscle	16.0 ± 1.4	10

The results of the simultaneous bromide and iodide space determinations may be seen in Table 7. The results are the mean values from nine toads (*Bufo bufo*). Fig. 7 shows the relationships between the different halide spaces in the heart and skeletal muscle. The bromide and iodide spaces were determined simultaneously, while the chloride spaces were determined on other toads.

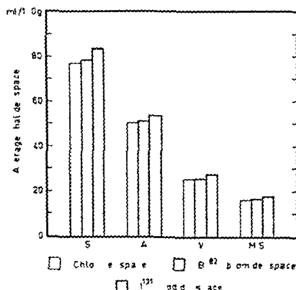


Figure 7. Mean values of halide space determinations (ml/100 g wet tissue) in heart and skeletal muscle. Br^{82} bromide and I^{131} iodide spaces determined simultaneously. Chloride spaces determined on other toad caught at the same time period.
S = sinus venosus, A = atrium, V = ventricle, MS = sartorius muscle

Table [illegible]

The Br^{82} bromide and I^{131} iodide spaces in the heart and skeletal muscle of newly caught toads (*Bufo bufo*). The determinations were made simultaneously. The table shows the mean values ± one standard deviation

Preparation	Br^{82} bromide space: ml per 100 g wet tissue	Br^{82} bromide space: No. of determinations	I^{131} iodide space: ml per 100 g wet tissue	I^{131} iodide space: No. of determinations
Sinus venosus	78.4 ± 2.3	[illegible]	83.[illegible] ± [illegible]	[illegible]
Atrium	[illegible]	[illegible]	[illegible]	[illegible]
Ventricle	[illegible]	[illegible]	[illegible]	[illegible]
Sartorius muscle	[illegible]	[illegible]	[illegible]	[illegible]

DISCUSSION

Determination of chloride in tissue

The method was found to give reproducible values. In burning the preparation it was probable that all chloride became available for analysis, including that situated intracellularly. The method has not, however, been compared with any other method, e.g. the isotope dilution method, and the possibility cannot therefore be precluded that some chloride may have disappeared during the actual preparation of the samples. By burning the samples (including the plasma samples) any interference of proteins on the mercurimetric titration was avoided. Phosphate can influence the end point of the mercurimetric titration, but DANIELSON (1963) found that this effect does not hold at pH = 3.5 and he also showed that there was a 100 % recovery of the amount of chloride added. In preparing the samples it is important that a large excess of calcium acetate solution is added and this is allowed to evaporate slowly in order to avoid chloride losses during the burning. Losses may have occurred during the sample preparation and burning, but since the values for the different halide spaces are in such good agreement it is improbable that any large loss occurred during the sample preparation.

The physico-chemical condition of chlorine in the tissue

Chlorine in the tissues is probably mainly in the ionized form. This ion has a very weak affinity for proteins. There

are a few reports that chloride ions bind to proteins in biological fluids and tissues (PETERS and VAN SLYKE 1931) and binding to plasma proteins is presumably small owing to competition with other substances with a higher affinity for proteins e g fatty acids amino acids organic acids and other proteins (COTLOVE and HOGBEN 1962)

Evidence of protein binding of chloride has only been obtained in relatively simple systems which have contained a purified protein and a single salt (KLOTZ 19[illegible]3) It has been shown that the binding affinity of chloride to serum albumin is approximately 1/4 of that of iodide Serum globulin binds less chloride per unit weight than serum albumin (CARR 1953)

It is thus probable that chloride exists mainly as a free diffusible ion

The chloride content in different tissues

The chloride concentration varied in different regions of the heart *with the highest value in the sinus venosus followed* by the atrium and ventricle in that order The average chloride concentration in the whole heart was higher than that of the sartorius muscle Chloride has principally the same distribution as sodium (see chapter III) but the total molar concentration of sodium is higher than that of chloride in both the heart and skeletal muscle in toads caught at the same time of year (cf Table 8) It is concluded that the greatest part of the sodium chloride in the heart and skeletal muscle is extracellular since in animal experiments only a very small part has been found

Table 8

The ratio of Na Cl (of molar concentration) in the heart skeletal muscle and plasma of newly caught toads (*Bufo bufo*) The table shows the mean value ± one standard deviation (calculated as the standard deviation for a ratio)

Preparation	Na Cl
Sinus venosus	1 1 ± 0 [illegible]
Atrium	1 3 ± 0 2
Ventricle	1 6 ± 0 3
Sartorius muscle	1 6 ± 0 4
Plasma	1 3 ± 0 1

to exchange slowly (AMBERSON *et al* 1938) and since radio active chloride and sodium ions penetrate the entire chloride and sodium spaces in rat heart in less than 8 minutes (MANERY and HAEGE 1941 MANERY and BALE 1941)

It may be assumed that part of the electrolytes are in connective and fat tissue It has been reported in dogs that about 30 % of all chloride in the muscle is in connective tissue and about 15–20 % in the rat and cat muscle (COTLOVE and HOGBEN 1962) The greatest part of this chloride should be easily exchangeable (AMBERSON *et al* 1938 WOODBURY 1956) while about 2 % of the total chloride in cat muscle exchanges slowly (COTLOVE and HOGBEN 1962) According to MANERY (1961) loose connective tissue has approximately the same chloride concentration (related to the tissue water) as plasma According to LOWRY GILLIGAN and KATERSKY (1941) the connective tissue constitutes a small but constant part of the heart and skeletal muscle tissue and the quantity of electrolytes in this tissue is therefore probably small compared with the quantity localized in the remaining part of the extracellular spaces of muscle tissues It is known from histological evidence that connective tissue is relatively abundant in the atrium and the sinus venosus and thus the total electrolyte content of these tissues may therefore be relatively large Any sodium and chloride which is bound in the connective tissue must however be easily exchangeable since the sodium and bromide spaces very rapidly reach their maximal values (cf chapter IX)

According to MANERY (1961) fat tissue also has approximately the same chloride concentration as plasma (in terms of tissue water) COTLOVE (1963 *a*) found that neutral fat contained only 0 3 % of the total chloride content in the skeletal muscle of the rat As is evident in chapter II the fat extractable with petroleum ether is a very small part of the wet weight in the tissues investigated

Comparisons between different halide spaces in the same tissue

The extracellular volume in muscle as estimated by the relative area of the space between the fibres in histological

sections corresponds approximately to the chloride space and was one of the reasons that the chloride space became used as an approximation for extracellular space (FENN 1936) Stable or radioactive bromide have been used similarly for the determination of bromide spaces in different tissues The distribution of bromide greatly resembles that of chloride in most tissues (WEIR and HASTINGS 1939 WALLACE and BRODIE 1939 GAMBLE *et al* 1953)

The results of the space determinations for the different halides show that these do not definitely differ significantly (in all cases $P > 0.02$) for any particular tissue Despite certain physico chemical dissimilarities between the ions (see further chapter VII) the distribution appears on the whole to be the same for the tissues investigated here

Comparisons between halide spaces in different tissues

There are several possible explanations for the differences in the size of the halide spaces between the different tissues (sinus > atrium > ventricle > skeletal muscle) If firstly the halide spaces are assumed to be approximations for the extracellular space the variations in size of the spaces should indicate that the size of the extracellular space varies in different regions of the heart and that the heart has a larger extracellular space than the skeletal muscle Secondly the intracellular halide concentration may vary in different tissues In order to assess in more detail these possibilities studies have been made on the distribution of other substances which to the greatest part are distributed in the extracellular space With the aid of these extracellular space approximations it is possible to assess more accurately the size of the extracellular space and also calculate the intracellular electrolyte concentrations if the total electrolyte content of the tissue and the plasma concentrations are known

For a discussion of the *length of the distribution time* see further chapter VII

CHAPTER V

The extracellular space in the heart and skeletal muscle

The electrolyte content of the intracellular phase must be calculated as the difference between the total tissue electrolyte and the electrolytes present in the extracellular space (water and connective tissue). For determination of the latter the volume of the extracellular space must be known, its electrolyte concentration being calculated from the corresponding plasma values. Correct evaluation of the extracellular space is especially important in the case of such ions as sodium and chloride whose intracellular content is low.

The extracellular space in a tissue sample is conventionally calculated from the total tissue content of a molecular or ionic species which is regarded as being distributed only throughout the extracellular space without entering the specific cell phase (e.g. muscle fibres). There appears to be no substance ideal for this purpose. The extracellular indicator should have the following properties: (a) it must not be metabolized, (b) it must not enter the specific cells, i.e. it must be too large or have a structure preventing this, and (c) it must distribute throughout the solvent in the extracellular space as in plasma, since its concentration in the latter is used as the reference value. This means that it should not become fixed (e.g. adsorbed) in any extracellular site. The extracellular space is, however, a complex structure containing in addition to cellular elements various solid structures, i.e. fibrillar (see chapter I), and also the high polymer hyaluronic acid so that its physico-chemical properties are complicated (MEYER *et al.* 1956; ROGERS 1961; OGSTON and SHERMAN 1961). The choice of an extracellular indicator is therefore somewhat arbitrary and

often a non-electrolyte e g mannitol or inulin is chosen These molecules are highly water soluble, neutral and are not known to undergo any specific interactions in the extracellular space As mentioned in chapter I various electrolytes resembling the chloride and sodium ions in their distribution have also been used for this purpose

The aim of this investigation was therefore to study the distribution of certain substances which it is believed remain essentially extracellular and to compare their calculated distribution spaces with the sodium and halide spaces

METHODS

The distributions of C^{14} mannitol C^{14} sucrose C^{14} maltotriose (three 1–4 α linked glucose units) and S^{35} sulphate in the form of carrier free $Na_2S^{35}O_4$ were studied in different series of toads kept for 5–7 months since capture Approximately 1 ml normal Ringer solution containing about 5 μC of the radioactive substance was injected into the posterior lymph sac of each toad after which the labelled solutes were allowed to distribute for different lengths of time (15 mins to 50 hours) At the end of the distribution period the toads were pithed and tissue and blood samples were collected, after which the plasma and tissue samples were prepared and analysed for their radioactive contents as described in chapter II

The different spaces were calculated in terms of 100 g wet tissue in the same way as for the halides spaces (cf chapter IV page 40) using also the same values for the water and protein content of plasma (see page 40)

RESULTS

The C^{14} mannitol spaces

Figs 8 and 9 show the C^{14} mannitol spaces (mol wt = 182) in the heart and skeletal muscle of the toad in relation to the distribution time The space in the sinus venosus is of the order of 60 ml 100 g tissue after distribution periods of 0 5–10 hours but increases after longer distribution periods to about 70 ml The mannitol space in the atrium is about 40 ml 100 g tissue after distribution period of 1/2–10 hours reaching

after about 35 hours values about 55 ml/100 g tissue i.e. of the same order as the Br^{82} bromide space (cf. chapter IV, page 13 or chapter VI, page 60). This progressive increase probably means that the mannitol slowly penetrates the cell. In the ventricle the mannitol space is smaller than of Br^{82} up to a distri-

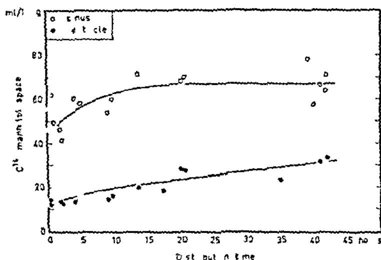


Figure 8. C^{14} mannitol spaces (ml/100 g wet tissue) in the sinus venosus and ventricle after different distribution periods. (The curves are drawn by eye)

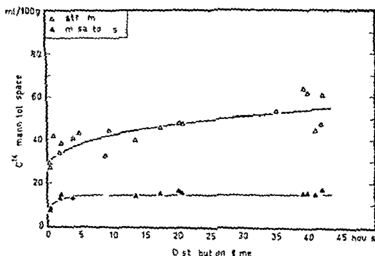


Figure 9. C^{14} mannitol space (ml/100 g wet tissue) in the atrium and sartorius muscle after different distribution periods. (The curves are drawn by eye)

bution period of about 20 hours, but after longer periods increases considerably reaching values of about 35 ml/100 g wet tissue after a distribution period of 40 hours compared with 15 ml after a shorter period (0.5–10 hours). For skeletal muscle (sartorius) the mannitol space is about 14 ml/100 g wet tissue and remains the same even after as long a distribution period as 40 hours. Table 9 shows the C^{14} mannitol determinations where the radioactive substance was allowed to distribute for 0.5–9.5 hours.

Table 9

The C^{14} mannitol spaces in the heart and skeletal muscle of the toad (*Bufo bufo*). The table shows the mean values ± one standard deviation for determinations where C^{14} mannitol had distributed for 0.5 – 9.5 hours and also the number of determinations

Preparation	ml/100 g wet tissue	No. of determinations
Sinus venosus	[illegible]4.2 ± [illegible]	8
Atrium	2[illegible].1 ± 4.6	[illegible]
Ventricle	14.[illegible] ± 1.8	8
Sartorius muscle	14.2 ± 1.4	6

The C^{14} sucrose spaces

Figure 10 and Table 10 show the results of the determinations of the C^{14} sucrose spaces (mol wt = 342) in the heart and skeletal muscle. Determinations were made on six toads. For the sinus venosus a mean value of 61.1 ml/100 g wet tissue was obtained for five determinations made with a distribution period of less than 14 hours, which is of the same order of size as the corresponding mannitol space. After a distribution period of 40 hours the sucrose space in the sinus venosus had further increased to about 7[illegible] ml/100 g tissue (one determination). For the atrium a mean value of 42.3 ml/100 g wet tissue was obtained for a distribution period of less than 14 hours, while after 40 hours the value had increased to 70 ml/100 g tissue. For the ventricle a mean value of 1[illegible].4 ml/100 g tissue (two determinations) was obtained for distribution periods of less than 4 hours, but after about 13 hours it had increased to approximately 2[illegible] ml/100 g tissue and after 40 hours to

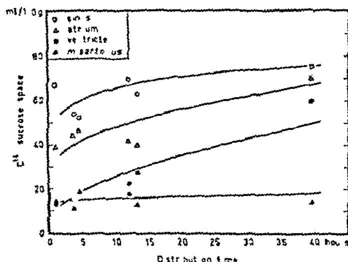


Figure 10 C^{14} sucrose spaces in the heart and sartorius muscle (ml/10 g wet tissue) after different distribution periods (The curves are drav by eye)

Table 10

The C^{14} sucrose spaces in the heart and skeletal muscle of the toad (B *bufo*) The table shows the mean value ± one standard deviation

Preparation	ml/100 g wet tissue	No of deter minations	Distributi time in h
Sinus venosus	61 1 ± "	5	1 — 14
Atrium	42 3 ± 3 1	5	1 — 14
Ventricle	15 4 (13 8 16 9)	2	1 3 5
Sartorius muscle	14 8 ± 2 8	6	1 — 39

60 ml/100 g tissue For skeletal muscle (sartorius) a m value of 14 8 ml/100 g wet tissue was obtained (1–14 hou and no increase was observed even after a distribution pe as long as 40 hours

The C^{14} maltotriose spaces

C^{14} labelled maltotriose (mol wt = 504) was also use determine the extracellular volume in the heart and ske muscle in the toad Determinations were made on six to The results (calculated as for the other spaces see Fig show that C^{14} is concentrated in the tissues especially in

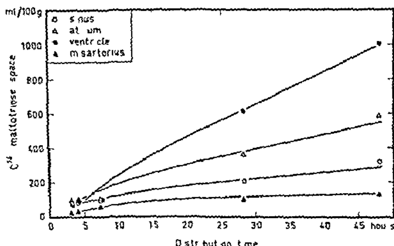


Figure 11 C^{14} maltotriose spaces in the heart and sartorius muscle after different distribution periods (the results calculated analogously with other spaces) *Note the scale of the ordinate*

heart as a consequence of which the values for the C^{14} maltotriose spaces are impossibly high This is presumably due to the breakdown of the maltotriose either during storage (the maltotriose had a rather high specific activity) before use or by the tissues themselves with the result that metabolised fragments penetrated and accumulated in the cells The greatest accumulation of C^{14} was in the ventricle followed by the atrium and the sinus venosus The heart as a whole had a higher content of C^{14} than the skeletal muscle

The S^{35} sulphate spaces

The results of the determinations of the sulphate spaces in the heart and skeletal muscle tissue are shown in Figs *12* and *13* *It may be seen that the sulphate ions rapidly distribute within a space which then increases only insignificantly after a long distribution period* The mean values of the results of the sulphate space determinations are given in Table 11 and these values refer to all toads in which the radioactive sulphate had been distributed for more than 50 minutes It is known that the sulphate concentration in the tissue equilibrated very rapidly (within a few minutes) with the plasma (see HUTTER 1961 SJÖSTRAND 1964 *inter alia*) and the rapidity with which

the maximal values were reached fit well with the hypothesis that the sulphate ions are limited to the extracellular space It is possible however that a small fraction of the sulphate ions also penetrate the cell membrane but this is so small that it can be disregarded in calculating the sulphate space

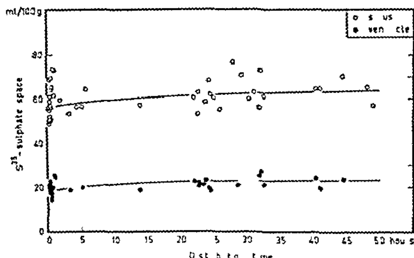


Figure 12 S^{35} sulphate spaces (ml 100 g wet tissue) in the sinus venosus and ventricle after different distribution periods (The curves are drawn by eye)

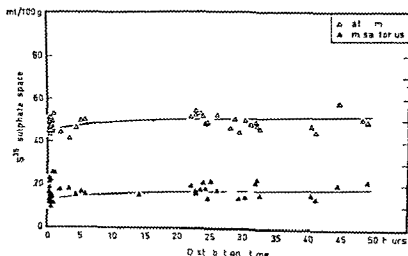


Figure 13 S^{35} sulphate spaces (ml 100 g wet tissue) in the atrium and sartorius muscle after different distribution periods (The curves are drawn by eye)

Table II

The S^{35} sulphate spaces in the heart and skeletal muscle of the toad (*Bufo bufo*). The table shows the mean values ± one standard deviation for toads in which the radioactive sulphate was allowed to distribute for more than 60 mins

Preparation	ml/100 g wet tissue	No. of determinations
Sinus venosus	62.8 ± 6.[illegible]	29
Atrium	48.8 ± 4.0	28
Ventricle	22.0 ± 2.5	30
Sartorius muscle	17.2 ± 2.6	28

DISCUSSION

The C^{14} mannitol spaces

PACE (1962) and many other authors have used the mannitol space as an indicator for the extracellular space. After short distribution periods there is nothing to indicate that the mannitol penetrates the cell membrane. The experiments reported here indicate that whereas in sinus venosus and skeletal muscle apparently steady values of the calculated space were attained (striated muscle in 5 hours, sinus venosus in 15 hours) the C^{14} activity continued to rise in atrium and ventricle over a long period (≈ 45 hours). The increase was most marked in the ventricle where after about 40 hours the mannitol space was about 30 % greater than the bromide space.

The question arises as to the cause of the slow secondary increase in C^{14} activity. There seems to be no reason to doubt the radiochemical purity of the solute. Radiochromatography some months before the experiment (butanol acetic acid water 4 : 1 : 2 or iso propanol pyridine water acetic acid 8 : 8 : 4 : 1) indicated high purity, and gel filtration (shortly after these experiments) through a tightly cross linked gel (Sephadex®) gave no evidence of heterogeneity (MARSDEN, *personal communication*). The gel filtration method seems to be a rather sensitive test of homogeneity provided the impurities have a different molecular weight and structure from the main component. A further indication of adequate radiochemical purity is the rapid attainment of a steady level in striated muscle. It would be hardly likely that diverse impurities were penetrant only in cardiac and not in skeletal muscle.

The shape of the radioactivity time curves indicates that at least two compartments probably exist It seems reasonable to locate the first rapid compartment in the extracellular space The second slow compartment may be (a) a result of heterogeneity in the extracellular space i e due either to a more slowly exchanging solvent region or to some fixation process such as adsorption or (b) due to entry of the solute into the intracellular space The question of adsorption cannot be settled directly but there is no evidence that the physico chemical properties of the tissue should provide adsorption sites for a polyhydric alcohol though considerable weak hydrogen bonding interactions may be expected Further there is the discrepancy in behaviour between sinus venosus and striated muscle on the one hand and atrium and ventricle on the other It seems here more reasonable to attribute these differences rather to the specific cells of the tissue than to the intercellular matrix Thus permeation into the intracellular space appears the more likely explanation for the slow secondary rise This finds some support in the fact that the calculated C^{14} spaces in ventricle and atrium are larger than the corresponding bromide spaces after a long distribution period Mannitol does not appear to penetrate into striated muscle and in view of this it is not surprising that the permeation which probably occurs in cardiac muscle should be rather slow

The C^{14} sucrose spaces

Uptake of C^{14} activity in all the heart tissues continued throughout the distribution periods and steady values were not obtained Striated muscle on the other hand rapidly (<5 hours) attained a steady value The radiochemical purity was not tested but using the striated muscle steady value as an index this seems to have been satisfactory (see Mannitol discussion) The failure of the C^{14} uptakes to reach steady values in the heart samples suggests that either the C^{14} sucrose or some metabolic products derived from it entered the cells

PAGE (1962) in *in vitro* experiments on cat heart muscle found that the smallest molecule that could not penetrate the cell membrane was mannitol (calculated molecular radius =

= 4.0 Å) in distribution periods of up to two hours. For comparison it may be mentioned that sucrose has a molecular radius of 4.5 Å (PAGE 1962).

From the present experiments it seems that a relatively long period is required in the heart for intracellular equilibration of C^{14} labelled solutes in plasma, since in 40 hours the sucrose spaces had not reached values of the same order of size as the total water volume. In skeletal muscle C^{14} activity appears to remain in the extracellular space, although the possibility cannot be excluded that exceedingly slow penetration into the muscle cells does occur.

The C^{14} maltotriose spaces

Since certain cells, e.g. erythrocytes (ÖSTLING 1964) are impermeable to maltotriose (mol. wt = 504), at least *in vitro*, this substance was tested for determining the ECV in the heart, but was found unsuitable. The results (see Fig. 11) showed an accumulation of C^{14} in the heart which was especially pronounced in the ventricle. The enzyme maltase, which is found in the intestine in mammals (WHITE *et al.* 1954) and probably also in toads, presumably splits the maltotriose with the formation of C^{14} glucose or other derivatives, which can enter the cell either passively or actively. Penetration of maltotriose itself is hardly likely, because of the size of the molecule (PAGE 1962).

The S^{35} sulphate spaces

Sulphate ions have also been used for determining the extracellular space (for the heart muscle see JOHNSON 1955, MIDDERGERKE 1963; for skeletal muscle see WALSER *et al.* 1954). The sulphate space has been considered to be somewhat larger than those of mannitol, sucrose and inulin. This is probably because sulphate also enters the extracellular connective tissue, in contrast to the carbohydrate molecules, which are not considered to penetrate the connective tissue (SELKURT 1963). It is evident from the results (see Figs. 12 and 13) that the sulphate spaces rapidly reach a maximum, after which the size

remains unchanged after long distribution periods. If some of the sulphate ions do enter the connective tissue in the ECV (as claimed by SHEATZ and WILDE 1950 among others) the cells must be very permeable to them since the sulphate space reaches its maximal value so rapidly. The specific heart cells on the other hand appear almost completely impermeable and if there is any sulphate penetration it must be very slow.

For more detailed comparisons between the sulphate, sodium and bromide spaces, see the discussion in chapter VI (cf. COTLOVE and HOGBEN 1962 and WALSER *et al.* 1951).

For a further discussion on the properties which determine the penetration capacity or permeability of cell membranes to non-electrolytes and ions, see chapter VII (discussion).

CHAPTER VI

Simultaneous determinations of the Na^{24} sodium, Br^{82}-bromide and S^{35} sulphate spaces in the heart and skeletal muscle

The aim of this part of the investigation was to determine the distribution of Na^{24}, Br^{82} and $S^{35}O_4$ in different regions of the heart and in the skeletal muscle *simultaneously* in order to compare their approximate extracellular spaces. All of these ion spaces have been used in the past as approximations of the extracellular spaces in different tissues. Sodium and bromide ions penetrate the cells to some extent and for this reason give an overestimate when used in the determination of the ECV. It is also supposed that sulphate ions enter cells (GAMBLE 1952, cf. also chapter V) but presumably to a small extent.

In the determination of stable sodium in the different regions of the heart there are certain practical difficulties, especially with regard to the sinus venosus, but with the aid of radioactive isotopes it is possible to make an accurate determination of the total content. If the radioactive isotope Na^{24} is allowed to equilibrate with stable sodium, the total content of stable sodium can be calculated from the specific activity of Na^{24} in the plasma and the total Na^{24} content in the tissue samples (assuming that the tissue specific activity is the same as that in the plasma). The results make possible a comparison with the determinations of the Na content in the heart and skeletal muscle reported in chapter III.

METHODS

Toads (*Bufo bufo*) which had been kept in captivity for just over 7 months were used for the experiments.

Approximately 1 ml radioactive Ringer solution was injected

into the posterior lymph sac in a series of 10 toads This solution contained 50 μC Na^{24} (specific activity 20 mC/g Na) 10 μC Br^{82} (specific activity 74 mC/g Br) and 5μC S^{35} (carrier free) per ml The isotopes were allowed to distribute in the toads for periods of 22–50 hours This period can be regarded as adequate for complete equilibration of Na^{24} and Br^{82} with stable isotopes both intra and extracellularly in the heart and skeletal muscle since MANERY and BALE (1941) found that sodium and chloride ions equilibrated the entire chloride and sodium space in the heart in less than 8 minutes Data for non electrolytes which penetrate the cells indicate that isotope efflux from a previously equilibrated muscle was virtually complete in less than 100 minutes (HOROWITZ and FENICHEL 1963)

At the end of the distribution period the toads were killed by pithing and blood and tissue samples were collected The sinus venosus and duplicate samples of atrium ventricle and skeletal muscle were weighed The tissue and plasma samples were placed on the special combustion papers used in preparation for liquid scintillation counting (see chapter II page 25) dried in an oven at about 90°C for two hours and then analysed for their contents of Na^{24} and Br^{82} by means of gamma spectrometry

When about 10 half lives of Br^{82} ($T_{1/2}$ = 35 9 hrs) had elapsed the samples were burned and analysed for their content of S^{35} by liquid scintillation counting (see chapter II)

Two plasma samples of 25 μl each were used for flame photometric determination of sodium (see chapter II)

RESULTS

The Na^{24} sodium Br^{82} bromide and S^{35} sulphate spaces

The results were calculated as described in chapters IV and V No Donnan correction was used but a correction was made for the solid content in the plasma The results showed that there was no further increase in the size of the Na^{24} sodium Br^{82} bromide and S^{35} sulphate spaces after a long distribution period Fig 14 shows the mean values for the different spaces The results clearly showed as also did previous extracellular

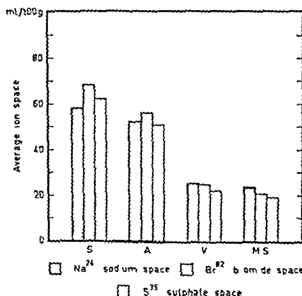


Figure 14 Mean values of simultaneous ion space determinations (ml/100 g wet tissue) in the heart and skeletal muscle
S = sinus venosus A = atrium V = ventricle MS = sartorius muscle

space determinations with $S^{35}O_4$ Br^{82} and I^{131} that the sinus venosus had the largest ECV followed by the atrium ventricle and skeletal muscle in that order *regardless of which substance had been used for determining the ECV*

Table 12 gives the results of the different ion space determinations The S^{35} sulphate spaces did not differ significantly from the Br^{82} bromide spaces in any of the tissues ($P > 0.01$

Table 12

The Na^{24} sodium Br^{82} bromide and S^{35} sulphate spaces in the heart and skeletal muscle in the (*Bufo bufo*) The determinations were made simultaneously The table shows the mean value one standard deviation and the number of determinations

Preparation	Na^{24} sodium space		Br^{82} bromide space		S^{35} sulphate space	
	ml per 100 g wet tissue	No of determinations	ml per 100 g wet tissue	No of determinations	ml per 100 g wet tissue	No of determinations
Sinus venosus	58 3 ± 3 4	8	68 3 ± 3 5	8	62 ± 6 6	10
Atrium	52 5 ± 3 7	9	56 3 ± 5 8	9	51 0 ± 3 9	10
Ventricle	25 5 ± 3 1	10	24 7 ± 2 2	10	22 2 ± 2 7	10
Sartorius muscle	23 7 ± 2 6	10	21 0 ± 3 2	10	19 3 ± 2 1	10

in all cases). The S^{35} sulphate space in the sartorius muscle was significantly smaller than the Na^{24} sodium space ($P < 0.01$) but no definite difference was observed in the other tissues (for the sinus and atrium $0.5 > P > 0.1$, for the ventricle $0.05 > P > 0.02$).

The Na^{24} sodium space in the sinus venosus was significantly smaller than the Br^{82} bromide space ($P < 0.01$), but in the other tissues investigated there was no clear difference ($P > 0.05$).

The total sodium content in different tissues

The specific activity of Na^{24} in the plasma was calculated on the basis of determinations of stable and radioactive sodium. The total content of sodium in the different tissues was calculated under the assumption that Na^{24} was completely equilibrated with stable sodium. The results are shown in Table 13. The values do not differ significantly from those found for stable sodium in the heart and skeletal muscle in toads which had been kept in captivity for corresponding periods (i.e. about 6 months, see chapter III, Table 3).

DISCUSSION

Ionic spaces

The fact that the Br^{82} bromide space is larger than the Na^{24} sodium space in the sinus venosus may be due *inter alia*

Table 13

The sodium content in the heart and skeletal muscle in the toad (*Bufo bufo*) calculated on the basis of the Na^{24} content in the tissues and the specific activity of Na^{24} in the plasma. (The Na^{24} was considered to be completely equilibrated with the stable sodium.) The table shows the mean values ± one standard deviation and the number of determinations.

Preparation	meqv/kg wet tissue	No. of determinations
Sinus venosus	[illegible]4 ± 4.0	8
Atrium	68.5 ± 6.8	9
Ventricle	33.3 ± 4.9	10
Sartorius muscle	30.[illegible] ± 3.2	10

to the bromide intracellular concentration being higher than the sodium or to differences in the electrolyte composition in the connective tissue which is found in abundance in the sinus venosus (see KISCH 1961 among others) In the other tissues investigated there were no apparent differences between the sodium and bromide spaces where it can be concluded that the distribution of bromide and sodium ions are probably on the whole similar

The S^{35} sulphate space in skeletal muscle is significantly lower than the Na^{24} sodium space probably due to the fact that the sulphate ions penetrate the muscle cells to a much smaller extent than sodium

No difference was observed between the Na^{24} sodium and S^{35} sulphate spaces in the sinus and atrium ($0.5 > P > 0.1$) and neither was there any definite difference in the ventricle It must be pointed out however that it is not easy to detect differences between distribution spaces for substances which are only present in the extracellular space and those which though mainly extracellular enter the intracellular compartment to a very limited extent especially if the ECV is large in relation to the intracellular volume e g as in the sinus venosus and atrium

The total sodium content

The values for the skeletal content of sodium in the heart and skeletal muscle determined with the aid of radioactive sodium conform well with the values found on analysis of stable sodium in toads kept for 6 months In comparison with toads kept for 8 months on the other hand the value for skeletal muscle differs significantly ($P < 0.01$) This difference cannot at present be explained

CHAPTER VII

ECV determinations and calculations of intracellular concentrations

This chapter discusses the values of the extracellular space obtained with different indicators and describes the calculations of the intracellular electrolyte concentrations. Factors of importance in penetration of ions and molecules into cells and through membranes are also discussed together with the question of a Donnan distribution between the plasma and extracellular water.

Comparison of ECV determinations

In calculating the distribution volumes of the different substances correction was made for the solute volume in the plasma (see chapter IV, page 40) but not for any possible Donnan effect between the plasma and the extracellular water.

The results of the space determinations are summarized in Figs. 15 and 16 and given more fully in Tables 6 (p. 42), 7 (p. 43), 9 (p. 50), 10 (p. 51), 11 (p. 54) and 12 (p. 60). The figures show the mean values for the different groups of determinations. In the figures there are three groups of columns for each tissue. The first groups represents the mean values of the halide spaces determined on newly caught toads and discussed in capter IV. It was shown there that there was no statistically significant difference ($P > 0.02$ in all cases) between the different halide spaces in any particular tissue.

In the second group the mean values for the C^{14} mannitol, C^{14} sucrose and S^{35} sulphate spaces are combined. The investigations in this group were made mainly during the same period (the toads had been kept for 5—7 months). The sulphate spaces were significantly greater ($P < 0.01$) than the cor

responding mannitol spaces for all tissues The sulphate spaces were also significantly greater than the corresponding sucrose spaces in the atrium and ventricle ($P < 0.01$) but not in the sinus and skeletal muscle ($P > 0.05$ and $0.05 > P > 0.02$ respectively)

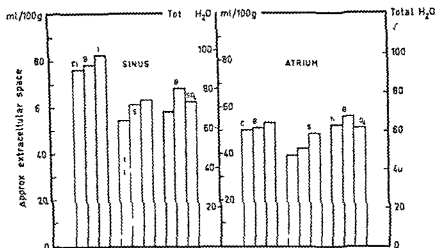


Figure 15 Mean values of space determinations made with different ions and molecules in the sinus and atrium For further details see text page 33
(Chloride spaces determined with stable isotopes other spaces determined with radioactive isotopes or radioactive labelled substances)

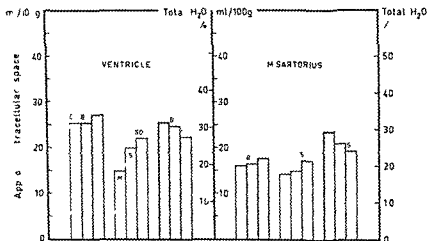


Figure 16 Mean values of space determinations made with different ions and molecules in the ventricle and sartorius muscle For further details see text page 63
(Chloride spaces determined with stable isotopes other spaces determined with radioactive isotopes or radioactive labelled substances)

The third group comprises simultaneous determinations of the Na^{24} sodium Br^{82} bromide and S^{35} sulphate spaces reported in Table 12 (p 60) The Na^{24} sodium and Br^{82} bromide spaces were significantly greater than the C^{14} mannitol and C^{14} sucrose spaces in the atrium ventricle and sartorius muscle ($P < 0\ 01$) The Br^{82} bromide space differed significantly from the mannitol space *($P < 0\ 001$) in the sinus venosus but not from the* sucrose space ($0\ 05 > P > 0\ 02$) No significant difference was observed between the Na^{24} sodium space in the sinus and the C^{14} sugar spaces ($P > 0\ 1$)

The Br^{82} bromide space in the sinus venosus and sartorius muscle in the first group differed significantly from the corresponding space in the third group ($P < 0\ 01$) but not in the atrium and ventricle ($0\ 5 > P > 0\ 1$) This difference cannot at present be fully explained but may be due to seasonal variations in the tissues

The water content in the heart and skeletal muscle

In order to calculate the intracellular volume the water content was determined according as described in chapter II page 20 The results are given in Table 14 The water content is highest in the sinus venosus followed by the atrium ventricle and skeletal muscle in that order *(significant dif*ference between the different tissues $P < 0\ 01$)

Table 14

The water content in the heart and skeletal muscle in toads The table shows the mean values ± one standard deviation

Preparation	Water content ml/100 g wet tissue
Sinus venosus	84 5 ± 1 1
Atrium	8 8 ± 1 6
Ventricle	81 2 ± 1 3
Sartorius muscle	80 0 ± 0 5

Intracellular sodium concentration

The intracellular concentrations of sodium and chloride were calculated from the experimental results on which Table 12 is based The following notation will be used

ECV = the extracellular volume (ml/100 g wet tissue)
$[Na^{24}]_m$ = cpm Na^{24} per 100 g wet tissue
$[Na^{24}]_p$ = Na^{24} concentration in plasma (cpm/l)
$[Na^{24}]_i$ = Na^{24} concentration in the intracellular water (cpm/l)
$[Na^{24}]_e$ = Na^{24} concentration in the extracellular water (cpm/l)
H_2O_m = water volume in the tissue (ml/100 g wet tissue)
$[Na]_p$ = sodium concentration in the plasma (meqv/l)
$[Na]_i$ = sodium concentration in the intracellular water (meqv/l)

The intracellular water volume (ICV) was calculated as the difference between the water volume and the ECV of the tissue determined with different substances. The intracellular sodium concentration $[Na]_i$ was calculated as the ratio $[Na^{24}]_i \cdot [Na]_p/[Na^{24}]_p$.

$[Na^{24}]_i$ was calculated from the determined molecular or ionic spaces according to the following formula

$$[Na^{24}]_i = \frac{1000 \cdot [Na^{24}]_m - ECV \cdot [Na^{24}]_e}{H_2O_m - ECV}$$

where $[Na^{24}]_e$ was calculated in the same way as $[Br^{82}]_e$ as described in chapter IV. The results are given in Table 15.

The intracellular Na concentration determined on the basis of the sulphate spaces (determinations made simultaneously with the Na estimations) *did not differ between the different heart regions and skeletal muscle* (sinus–atrium $0.5 > P > 0.1$, atrium–ventricle $P \approx 0.5$, ventricle–skeletal muscle $0.05 > P > 0.02$) and neither was there any significant difference when the mean values for the sulphate or sucrose spaces were used for the calculations (atrium–ventricle $P > 0.5$, ventricle–skeletal muscle $P = 0.1$). The mean value for $[Na]_i$ in the sinus was negative with these three methods of calculation (Table 15, lines 1, 2 and 4) *which must depend on the method of calculation.* The intracellular Na content is estimated as a difference between two almost equal numbers, which introduces a great uncertainty in the difference.

The intracellular Na concentration calculated by means of the mannitol spaces was higher in the atrium than in the

Table 15

Intracellular concentration of sodium [Na]i and chloride [Cl]i in the heart and [illegible] muscle [illegible] with the [illegible] different [illegible] molecular space. The table shows the mean values ± standard deviation for the following number of determinations [illegible] and ventricle and sartorius muscle 10

Extracellular space	[Na]i in mM/l				[Cl]i in mM/l			
	Sinus	Atrium	Ventricle	Sartorius muscle	Sinus	Atrium	Ventricle	Sartorius muscle
S^{35} sulphate space (det. simultaneously with Na and Br)	30	13 ± [illegible]	[illegible]	[illegible] 3	[illegible]	[illegible]	[illegible]	[illegible]
S^{35} sulphate space (mean value)	[illegible]	[illegible]	7 ± [illegible]	[illegible]				
C^{14} mannitol space (mean value)	16 ± [illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C^{14} sucrose space (mean value)	[illegible] ± 10.8	[illegible]	[illegible] ± 6.6	[illegible]	370	[illegible] ± 11.4	13 ± 3.[illegible]	[illegible]
Br^{82} bromide space (mean value)	[illegible] ± 4.6	[illegible] ± 1.4	[illegible] ± 8.[illegible]	8 ± [illegible]				
Na^{24} sodium space (mean value)					31 [illegible]	[illegible]	[illegible] ± 3.[illegible]	[illegible]

other tissues ($P < 0.01$) When the Br^{8} bromide space was used as an approximation for the ECV the [Na] became negative in all regions of the heart In the skeletal muscle on the other hand the [Na] calculated from the bromide and sulphate spaces did not differ significantly ($0.1 > P > 0.05$) but was significantly lower than that based on the carbohydrate spaces ($P < 0.01$)

In the atrium ventricle and striated muscle calculations based on the sulphate space gave a lower value of [Na] than when the carbohydrate spaces were used ($P < 0.01$) No definite differences were however found for the sinus ($0.05 >$ $> P > 0.02$) the variations for this tissue being rather large

Intracellular chloride concentration

The intracellular Br^{8} concentration $[Br^{8}]_i$ was calculated *on the basis of the different ionic or molecular spaces in the* same way as $[Na^{+}]$ Assuming that Br^{8} is distributed similarly to the chloride ions the intracellular chloride concentration [Cl] was calculated as $[Br^{8}]_i \cdot [Cl]_p/[Br^{8}]_p$ where $[Cl]_p$ is a mean value from the analysis of plasma from 10 toads (see chapter IV) The results are given in Table 15

The [Cl] *calculated from the sulphate spaces* is greater in the sinus than in the ventricle and skeletal muscle ($P < 0.01$) but is not significantly different from that of the atrium ($0.02 > P > 0.01$) The latter is higher than that of the sartorius muscle ($P < 0.01$) but is *not significantly different* from the sulphate based [Cl] of the ventricle ($0.02 > P > 0.01$)

The *mannitol based* [Cl] in the atrium and sinus is greater than that *in the ventricle and skeletal muscle* ($P < 0.01$) *There* are however neither significant difference between the [Cl] values in the atrium and sinus ($0.1 > P > 0.05$) nor between the *ventricle* and skeletal muscle ($0.05 > P > 0.02$)

The *sucrose based* [Cl] in the sinus does not differ significantly from that of the ventricle and atrium ($P > 0.5$ and $0.02 > P > 0.01$ respectively) the [Cl] value of the atrium is however greater than that of the ventricle and skeletal muscle ($P < 0.01$)

The sulphate based $[Cl]_i$ *is less than that calculated from the carbohydrate spaces in the atrium, ventricle and skeletal muscle* (P < 0.01). There are no significant differences as regards the sinus.

Comparison between intracellular sodium and chloride concentrations

The intracellular sodium concentration is not significantly different from the chloride concentration in the atrium and ventricle (P > 0.02 in all cases) when calculated from the sulphate or carbohydrate spaces with simultaneous determinations of Na and Cl (Br). In the sinus the $[Na]_i$ calculated from the mannitol or sucrose spaces is lower than the $[Cl]_i$ (P < 0.01) but there is no significant difference when the calculations are based on the sulphate space (0.05 > P > 0.02). In the skeletal muscle the intracellular content of sodium is higher than the chloride content, regardless of which extracellular space indicator is used (P < 0.01).

Intracellular potassium concentration

The intracellular potassium concentration $[K]_i$ was calculated from the extracellular space and the total potassium content (reported in chapter III, Table 3; toads kept in captivity for less than 3 months). The following notation will be used:

$[K]_m$ = meqs per kg wet tissue
$[K]_p$ = potassium concentration in plasma (meqs/l)
$[K]$ = potassium concentration in the extracellular water (meqs/l)
$[K]$ = potassium concentration in the intracellular water (meqs/l)

K is calculated according to the following formula:

$$[K] = \frac{[K]_m \; 100 - ECV \; [K]}{H_2O_m - ECV}$$

Table 16

Intracellular concentration of potassium [K] in the heart and skeletal *muscle calculated with the aid of different ionic and molecular spaces* and the total content of potassium (reported in Tables 2 and 3 chapter III) The table shows the mean values ± on standard deviation for the following number of potassium determination sinus atrium and ventricle 14 and skeletal muscle 20

Extracellular space	[K] in g/l Sinus	Atrium	Ventricle	Sartorius muscle
1 S^{35} sulphate space	104 ± 33	101 ± [illegible]	[illegible] ± 11	114 ± 8
2 C^{14} mannitol space	[illegible] ± 20	[illegible] ± 1	83 ± 9	109 ± [illegible]
3 C^{14} sucrose space	9[illegible] ± 33	8[illegible] ± 1[illegible]	[illegible]0 ± 10	110 ± 8
4 Na^{24} sodium space	8[illegible] ± 14	112 ± 23	10[illegible] ± 12	12[illegible] ± [illegible]
Br^{82} bromide space	138 ± 34	128 ± 3	101 ± 11	121 ± [illegible]

where [K] is calculated in the same way as $[Br^{s}]_i$ (see chapter IV) The results of the calculations are given in Table 16 The intracellular potassium concentration [K] calculated on the basis of the mannitol sucrose sulphate or sodium spaces is lower in the atrium and ventricle than in the sartorius muscle and the ventricle is also lower than the sartorius when the bromide space is used ($P < 0.01$) The intracellular potassium concentration in the sinus is lower than in the skeletal muscle when calculated from the mannitol or sodium spaces *($P < 0.01$) but not when calculated from the other spaces* ($P > 0.05$)

The [K] showed no differences in comparisons between different heart regions regardless of which substance was used for the ECV determinations ($P > 0.1$ in most cases but $P > > 0.02$ *in all cases*) except that with the calculations based on the sodium space the [K] in the sinus was lower than that of the atrium and ventricle ($P < 0.01$)

The mannitol based [K] is lower than the sodium and bromide [K] values in the ventricle and skeletal muscle and is also lower than the potassium concentrations calculated from the sulphate spaces in the atrium and ventricle ($P < 0.01$) There are no differences for the sucrose based [K] values in any of the tissues ($P > 0.05$)

The sinus $[K]_i$ values calculated from the mannitol or sucrose spaces are lower than that based on the bromide space ($P < 0.01$) but do not differ from the values calculated from the other spaces ($P > 0.1$).

On calculating the intracellular potassium concentration e.g. from the sulphate space in toads which had been kept in captivity for a long period (not reported in the table) it was found that the $[K]_i$ was lower in the atrium, ventricle and skeletal muscle ($P < 0.01$) but that there was no definite decrease in the sinus ($0.05 > P > 0.02$).

DISCUSSION

Comparison of ECV between different tissues studied

It is apparent that the sinus venosus has the greatest ECV followed by the atrium, ventricle and skeletal muscle, *regardless of which substance is used for the ECV determinations*. (There was no significant difference, however, between the mannitol and sucrose spaces in the ventricle and the corresponding spaces in the skeletal muscle, $P > 0.02$.)

Relationship between the histological findings and the calculated values of the spaces

The extracellular volume in the muscle, as assessed from the relative surface area of the space between the fibres in histological sections, corresponds approximately to the chloride space, and this was one of the reasons that the chloride space became used as an approximation for the ECV (FENN 1936). SANDOW (1939) calculated theoretically the space between tightly packed parallel cylinders with the same fibre diameter as the muscle fibres in the frog sartorius muscle as 15.1% of the entire muscle volume. This value was in good agreement with experimental values for the chloride space.

No similar comparisons between histological and physiological findings have been made in the heart. From histological investigations on the frog heart (KISCH 1961) and from data about the conduction system in mammals (see KAWAMURA 1961, among others), the conclusion may be drawn

that the ECV is large both in the sinus venosus and in the conduction system and is to a great part occupied by collagen fibres.

It is possible that *connective tissue* and *fatty tissue* constitute a source of error in determinations of the ECV since it is not certain that the test substances penetrate these tissues *to the* same extent as other parts of the ECV. The neutral fat content in the tissues studied was about 1 % of the weight of the wet tissue (see Table 1 chapter II) and has therefore been considered unimportant. The connective tissue content has not been investigated but may possibly be of some significance at least in determinations of the ECV in the sinus venosus since it is known that this tissue is rich in connective tissue (KISCH 1961).

One possible explanation for the fact that the distribution spaces for the carbohydrate molecules are smaller than the corresponding ionic spaces is according to SELKURT (1963) that it is only with difficulty that mannitol and sucrose penetrate the connective tissue which should mean that these *spaces are smaller than the actual extracellular volume. If the* matrix of collagen fibres which pervades the extracellular space contains high polymers such as hyaluronic acid or other macromolecules *it is possible that some steric exclusion may* occur if large test molecules are used to indicate the ECV. Thus inulin (average mol wt ≈ 5000) may not have access to as large an extracellular space as a smaller molecule such as mannitol (OGSTON and SHERMAN 1961) (see also discussion p. 79).

It is evident that the absolute values for the intracellular *concentrations are strongly dependent on the size of the extra*cellular volume i.e. it is of great importance to use the measurement for the space which is *most representative for the physiological extracellular space* (cf. formulae on pages 66 and 69).

In determinations of the ECV in the heart the sulphate space has been assessed as the most correct approximation since its distribution volume rapidly equilibrates after which the volume does not appear to increase even after a long period. It has further been considered by other authors (see

PAGE 1962 *inter alia*) that this ion remains essentially extracellular PAGE (1962) showed that the volume of the heart muscle cells did not increase but remained constant when the K concentration was raised isosmotically—by exchange of the Na ions—in the bath solution the only anion of which consisted of impermeable sulphate ions When corresponding experiments were made with the permeable chloride ion the intracellular volume increased greatly PAGE interpreted these experiments as indicating that the sulphate ions remained extracellular

It is probably less suitable to use the mannitol space for example as an approximation for the LCV since it is claimed that mannitol penetrates the connective tissue only very slowly (SELKURT 1963) and since the distribution volume of mannitol becomes very large after a long period (see chapter VI) which probably indicates permeation into the cells

The water content

The water content is highest in the sinus venosus followed by the atrium ventricle and skeletal muscle in that order It is remarkable how little the water content varies among different preparations

Factors of significance in the permeation of solutes into cells

The transport of molecules or ions into living cells or through membranes is determined by several factors such as their concentration mobility and driving forces (TEORELL 1953) Several factors influence the permeability of a membrane to a molecule or ion namely the properties of the molecules or ions themselves (e g size hydration solubility charge) and properties of the membrane (pore size physico chemical structure charge affinity for solvents etc) The driving force may be a concentration gradient electrical potential gradient or a hydrostatic pressure gradient (TEORELL 1953) The electrical potential gradient does not influence directly undissociated or uncharged species

Properties of the molecules or the ions themselves

The properties which influence the permeability to molecules include the size, hydration and solubility in the membrane. Of the carbohydrate molecules used, the calculated molecular model radius of mannitol is 4.5 Å. (For a comparison of the radii of small molecules determined in different ways see SCHULTZ and SOLOMON, 1961.) (Maltotriose is certainly considerably greater but may break down in the tissue.) The carbohydrate molecules used may be able to penetrate the membrane in several principally different ways, i.e. by means of pre-formed pores, by solution in the membrane or by other chemico-metabolic processes. Of the molecules used, mannitol and sucrose pass through the endothelial membrane between the heart lumen and the extracellular space with ease, while penetration of the cell membrane takes place considerably more slowly (PAGE, 1962).

PAGE (1962) found that sucrose and mannitol (molecular model radii 4.5 Å and 4.0 Å respectively, see SCHULTZ and SOLOMON, 1961) were the smallest molecules which appeared to remain extracellular, at least in the heart muscle in the cat. Experiments reported here show that the mannitol and sucrose spaces became very large after long periods of distribution, at least in the atrium and ventricle, which would seem to indicate some penetration of the cell membrane.

The sizes of the ions used for determinations of approximate extracellular spaces (ionic spaces) are shown in Table 17.

Table 17

The crystal and hydrated radii of different ions [illegible] from the Stokes-Einstein [illegible] r = [illegible] radius, r_H = hydrated radius. Data [illegible] from NIGHTINGALE ([illegible])

Ion	r Å	r_H Å
[illegible]	[illegible]	[illegible]
K⁺	1.33	3.31
Cl⁻	1.81	3.32
[illegible]	[illegible]	3.3
[illegible]	2.1[illegible]	3.31
[illegible]	2	3.[illegible]9

where both the crystal and the so called hydrated radii are given. These radii are calculated from the behaviour of ions in solution and are somewhat controversial (see SAMOILOV 1957). The hydrated radii of the different halide ions are very similar but somewhat smaller than the corresponding radii of the sodium and sulphate ions. The calculated sizes of the ions and molecules used are compared in Fig. 17.

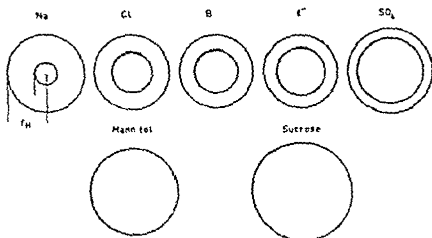


Figure 17. The sizes of the ions and molecules used for the ECV determinations. r_x = crystal radius, r_H = hydrated radius.

Properties of the membrane

The extent to which the different ions penetrate the cell membrane depends not only on the porosity (i.e. some function of the structure and size of the penetrant) but also on the membrane charge. There are reasons for believing that the heart cell membrane is negatively charged, the negative ions thus being retarded (see TEORELL 1953).

Driving forces

For both ions and molecules the chemical potential gradient is also any presence of a hydrostatic pressure gradient influences the distribution between the extra- and intracellular

spaces For ions in addition there is the influence of the electrical potential gradient For more detailed studies of the permeability of natural membranes and of the laws which determine the distribution of ions and molecules in biological systems reference may be made to DAVSON and DANIELLI (1952) TEORELL (1953) and HARRIS (1960) *inter alia*

The importance of the distribution period

As shown by the results reported in chapter V it is seen that the sulphate spaces very rapidly reach their maximal values while the mannitol and sucrose spaces continue to increase for long periods at least in the atrium and ventricle and exceed the corresponding halide spaces This may be interpreted as indicating that the sulphate ion remains extracellular or if it does penetrate the cell it does so to only a very small extent while the carbohydrate molecules slowly enter the cell

Other ions used (sodium and halide ions) equilibrate rapidly with the stable isotopes (see chapter IX and also MANERY and BALE 1941)

The importance of the Donnan factor

The extracellular fluid is assumed to be an ultrafiltrate of plasma the electrolyte composition of which can be calculated if correction is made for the water content of the plasma and for the Donnan equilibrium which is assumed to prevail between the plasma and the extracellular fluid

The theoretical Donnan factor for the distribution of univalent negative ions between plasma and protein free ultrafiltrates in man has been calculated as approximately 0 96 For the *chloride* and *sodium ions* there was good agreement between the predicted and observed values (VAN SLYKE WU and MCLEAN 1923) MANERY (1954) compared values for the Donnan factor (r) from the literature (obtained by ultrafiltration *in vitro* or from simultaneous electrolytic determinations *in plasma* and *oedema fluid*) This author considered that the factor r = 0 95 should be used for all negative ions

For positive ions (e.g. Na and K) the Donnan factor is somewhat greater than unity while the correction factor for plasma water is somewhat less than unity and these factors therefore tend to cancel each other out (see chapter IV page 11) whereby the concentration of positive univalent ions in the extracellular water may be assumed to be the same as in the plasma (cf BERGSTRÖM 1962).

For negative ions both the Donnan and the correction factor for the plasma water act in the same direction which means that the concentrations of negative ions are higher in the extracellular water than in the plasma. For the divalent sulphate ion the Donnan factor ($\sqrt{r_{SO_4}}$) should theoretically be r_{Cl}^2 i.e. of the order of $0.96^2 = 0.92$ if the sulphate ion were the predominant diffusible anion and if conditions were otherwise the same (DAVSON 1956).

For comparison it may be mentioned that SWAN, FEINSTEIN and MADISSO (1956) found that the equilibration concentration of radioactive sulphate in serum was 0.95 that of the sulphate concentration in a dialysate of human serum and that the corresponding value for dog serum was 0.96. The corresponding factors for chloride were 0.99 and 0.98 respectively. Cellophane membrane was used for dialysis (this is weakly negatively charged). These experiments showed that the Donnan factor for the sulphate ions is $\approx r_{Cl}$.

Of decisive importance to the ion distribution between the plasma and extracellular water is the construction and charge of the membrane which separates the lumen from the extracellular space. This consist of a single endothelial layer under which there is a layer of collagenous connective tissue. As mentioned previously (chapter II) the toad heart lacks coronary vessels.

In the calculations made here no correction has been made however for any Donnan distribution of ions between the plasma and the extracellular water since the protein content in plasma is so low and there are considerably practical and theoretical difficulties in determining a correct value for the Donnan factor for the ions used. In order to make an accurate correction for the Donnan effect the properties of the membrane which divides the plasma from the extracellular space

PART II

CHAPTER VIII

Introduction

As mentioned in the introduction to part I much attention has been paid in recent years to attempts at explaining how the asymmetry of ions between the intra and extracellular phases occurs and can be maintained and also how excitability arises BOYLE and CONWAY (1941) put forward a hypothesis regarding the properties of the cell membranes to explain the ionic asymmetry and the resting potential in striated muscle The diffusion and active transport of substances through the cell membrane have been studied extensively by USSING (1949 1951) According to the theory of HODGKIN and HUXLEY (1952 *a–d*) the ion distribution at rest is maintained by an active outward transport of sodium ions from the cells combined with a low sodium permeability This theory based mainly on results from squid axons explains the resting potential as a result of the difference in concentration between the intra and extracellular spaces

According to TEORELL (1960) the electrochemical potential gradient together with a hydrostatic pressure gradient may give rise to a flow of water which in its turn should result in a separation of ions in the charged pores in the membrane According to this theory the water transport between the cellular and extracellular phase could play a large part in the occurrence of the electrical phenomena and ion dyssymmetry (TEORELL 1962)

In order to explain the depolarization of the cell membrane during an action cycle in an excitable cell it was assumed that the permeability of the cell membrane was changed so that the sodium permeability became much greater than the potassium permeability (HODGKIN and HUXLEY 1952 *a–d*)

NOLLE (1960 1962) developed a theory which based on the

Hodgkin and Huxley theory and which took into consideration the *special physiological conditions in the heart tissues* A theoretical discussion on the sodium and potassium hypothesis has been presented by Brady and Woodbury (1960)

Numerous studies have been reported (see e g Brooks Hoffman and Orias 1955 Weidmann 1956 and Hoffman and Cranefield 1960) which support the assumption of a *relationship between the electrical activity and ionic flow in* excitable tissue Attempts have been made further with different models of oscillatory or instable systems to reproduce *the electrical phenomena which occur in excitable tissues* such as resting and action potentials the tissue's own rhythmicity etc. Common to all these theories is the importance of ion *transport By alteration of forces influencing the system or* the membrane attempts have been made to produce rhythmic changes in the system in order to simulate the natural conditions *in excitable tissues e g the effect of acetylcholine*

It has long been known that acetylcholine and vagal stimulation influence the ionic transport in the heart Howell and Duke *(1908) showed that vagal stimulation liberated potassium* ions into the perfusion solution which flowed through the heart Dale (1937) showed that Loewi's Vagusstoff (1921) *was the substance which was released on vagal stimulation* and which produced changes in the heart muscle Since this time the mode of activity of the acetylcholine released on *vagal stimulation has presented a great problem It has long* been known that the potassium ions are affected on vagal stimulation (Lehnartz 1936 Holland Dunn and Greig 1952 *a b*) Fatt and Katz (1951) put forward the hypothesis that the transmitter substance gave rise to a non specific increase of the ionic permeability of the cell membrane Burgen and Terroux (1953) presented experimental results which showed that acetylcholine acted on the heart muscle by changing the permeability to potassium ions which was confirmed by Sekul and Holland (1959) Harris and Hutter (1956) and Hutter (1961) among others Some investigations (Öbrink and Essex 1953 and Klein and Holland 1958) indicate that the acetylcholine or vagal stimulation influences the heart cell membrane's permeability not only to potassium

ions but also to sodium ions The concept now generally prevailing is however, that the acetylcholine or vagal stimulation give rise to a selective increase in permeability to potassium ions in the heart since few investigations support the view that the transport of other ions also is influenced by acetylcholine

The aim of this part of the investigations was *to study the electrolyte distribution in the vagus stimulated heart and to study the exchange of ions between the heart and blood on vagal stimulation in order to seek a basis for chronotropic changes* In the experiments the distribution of sodium potassium and chloride (bromide) in the vagus stimulated heart is compared with the distribution under normal conditions

CHAPTER IX

The sodium, potassium bromide and sulphate spaces in the heart on vagal stimulation

As mentioned in the introduction to part II acetylcholine is considered to act on the heart by inducing an increase in the potassium permeability of the cells Whether or not the transport of other ions e g sodium and chloride ions is also influenced by acetylcholine and vagal stimulation is somewhat problematical

The importance of chloride and other ions in the formation and propagation of impulses in excitable tissue is still relatively unclear It has long been considered that the anions are passively distributed (as a result of the distribution of the sodium and potassium ions HODGKIN & HUXLEY 1952 *a–d*) and thus do not contribute to any noteworthy extent to the resting potential TASAKI (1963) showed on intracellularly perfused squid axons that the permeability to a univalent anion Br^{82} was very small TASAKI and SPYROPOULOS (1961) showed that stimulation of the axon did not result in an increased flow of Br^{8} or other anions Complete exchange of intra and/or extra cellular chloride for sulphate or glutamate changed neither the resting nor the action potential in squid axons to any significant extent (TASAKI and SHIMAMURA 1962) These findings support the concept that the membrane of the axon has fixed negative charges which was postulated already in 1936 by TEORELL

CARMELIET (1961) found in Purkinje fibres that the chloride ions contributed very little to the resting potential but by resistance measurem[illegible] he found that during the action potential the chloride i[illegible] ntributed t[illegible] polarization by migrating into the c[illegible]

left aorta The sinus venosus atrium and ventricle were then excised together with a sample from the skeletal muscle (sartorius) The content of radioactive isotopes in the tissue samples was then determined

Vagal stimulation

In some of the toads in addition to the usual heart dissection one of the vagus nerves was dissected free approximately 1–1 5 cm from the heart—as a rule the right vagus nerve since this is somewhat larger than the left An electronic stimulator (Kistner lab stimulator Stockholm Sweden square pulse 15 volts frequency 20 per second duration 5 msec) and silver electrodes (distance between the electrodes about 2 mm) were used in the experiments YOUNG and UPHAM (1961) found that the longer the pulse duration used the longer was the cardiac arrest obtained The longest period of cardiac arrest was obtained with a stimulation frequency of 15–60 pulses per sec and a pulse duration of 6 msec

When the radioactive isotope had been allowed to distribute for the intended time the vagus nerve was stimulated and when cardiac arrest and subsequent vagal escape had been observed the heart was excised as quickly as possible The stimulation of the vagus nerve was continued while the tissue samples were being taken to avoid the possible disappearance of any effects of the stimulation on the distribution and transport of the ions in question The total number of stimulation pulses was 200–600 The procedure was otherwise the same as for the control series

Reference for the relative radioisotope concentration

In order to compare the results from different toads the ratio between the concentration of the radioactive isotope in the heart and the concentration in the sartorius muscle or in the plasma was determined i e muscle or plasma was used as a reference to express the *relative content* of the radioactive isotopes in the different regions of the heart

With short distribution periods ($<$ 1 hour) plasma seemed to be a better reference than skeletal muscle since the sartorius

muscle sometimes exhibits large variations in isotope concentration among different toads after the same short distribution period. DANIELSON (1962) for example concluded that vagal stimulation gave rise to a higher equilibration rate for Na^{24} since vagus stimulated hearts had higher Na^{24} contents relative to skeletal muscle than normal hearts after the same short distribution period. In these experiments it was probably the skeletal muscle reference which varied and which had a lower Na^{24} content in vagal stimulated than in normal animals (probably due to bad circulation in the vagus stimulated toads because longer handling time after pithing and before injection of the radioisotopes). The ratio between the radioactive isotope concentrations in the heart and plasma varied considerably less than the ratio between the concentrations in heart and skeletal muscle (in comparisons between different toads) even with short distribution periods and this reference was therefore used in most cases.

Experiments with Br^{82}

Approximately 1 ml normal Ringer solution containing 10–15 µC Br^{82} (74 mC/g Br) were injected into the posterior lymph sac of each of 40 toads. After various distribution periods (5 mins–19 hrs.) the toads were pithed and the heart dissected free. The sinus venosus, atrium, ventricle and a piece from the left sartorius muscle were then excised, blotted gently on filter paper, weighed and analysed for their contents of Br^{82} (as described in chapter II, page 22). Corresponding experiments were made with vagal stimulation of the heart (as described above).

Experiments with K^{42}

Approximately 1 ml radioactive Ringer solution containing 25 µC K^{42} was injected into the posterior lymph sacs of a number of newly caught toads. The isotopes were allowed to distribute for 10–32 hours, after which the toads were pithed and the heart dissected free. Heart and skeletal muscle samples were then taken—in part of the toads after vagal stimulation—

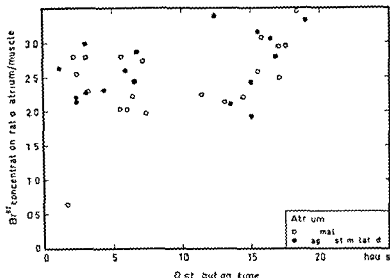


Figure 19 The relative Br⁸ concentration in the atrium (atrium/sartorius muscle) in normal and vagus stimulated toads (*Bufo viridis*)

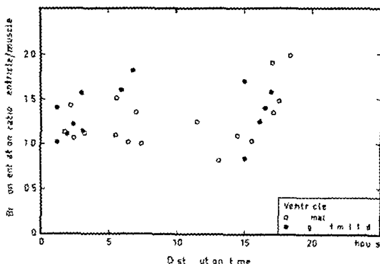


Figure 20 The relative Br⁸ concentration in the ventricle (ventricle/sartorius muscle) in normal and vagus stimulated toads (*Bufo viridis*)

The Br⁸ bromide Na or Na⁴ sodium and K⁴ potassium spaces in the heart and skeletal muscle

Figures 21 and 22 show the Br⁸ bromide spaces (i e the Br⁸ concentration in the heart relative to the Br⁸ content in

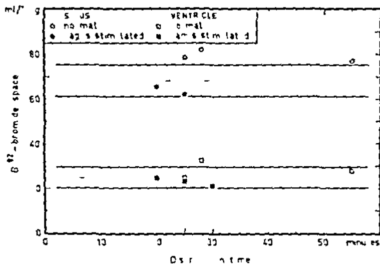


Figure 21 The Br[82] bromide spaces in the sinus venosus and ventricle in normal and vagus stimulated toads after short distribution periods The lines show the mean value $\pm$ 2 S D for the Br bromide spaces after distribution periods of 2?—50 hours (see chapter VI)

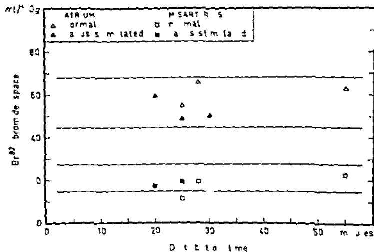


Figure 22 The Br[8] bromide spaces in the atrium and sartorius muscle in normal and vagus stimulated toads after short distribution periods The lines show the mean value $\pm$? S D for the Br[8] bromide spaces after distribution periods of ??—50 hours (see chapter VI)

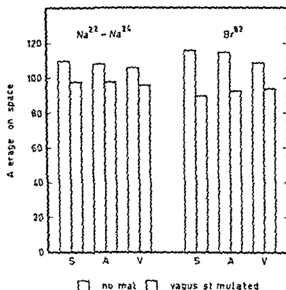


Figure 25 Mean values of Br⁸ bromide and Na Na²⁴ sodium spaces in normal and vagus stimulated hearts after short distribution periods (9—30 minutes) expressed as percentages of the mean value of the corresponding Br⁸ bromide spaces or Na⁴ sodium spaces found with distribution periods of 22—50 hours (see Table 12 page 60)

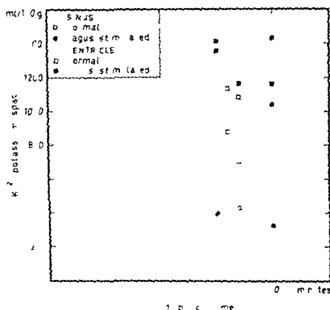


Figure 26 The K⁴ potassium spaces in the sinus venosus and ventricle in normal and vagus stimulated toads after short distribution periods

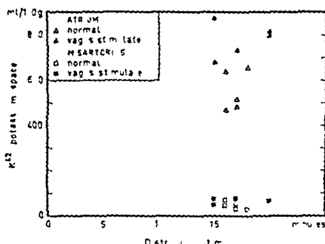


Figure . The K^{42} potassium spaces in the atrium and sartorius muscle in normal and vagus stimulated toads after short distribution periods

the normal hearts ($P < 0.01$) while in the sinus there is no significant difference between the normal and vagus stimulated hearts ($P > 0.10$)

No difference was observed in the K^{42} spaces in the skeletal muscle between normal and vagus stimulated toads ($P > 0.5$) Although vagus stimulation is associated with circulatory collapse it does not seem likely that this latter would produce an observable effect as it exists for so short a time Comparison of the K^{42} spaces in the skeletal muscle (sartorius) before and after vagal stimulation in the same toads showed no difference (these results are not given in the figure)

Relative K^{42} content in the heart after a long distribution period

Figures 28–30 show the relative K^{42} content in the heart (in relation to skeletal muscle) after a long distribution period (10–32 hours) There is no difference between normal and vagus stimulated hearts After this relatively long distribution period the K^{42} is probably almost completely equilibrated with stable easily exchangeable potassium in the heart and the relative K^{42} concentration in the heart should therefore be a

since the sulphate spaces which may be regarded as an approximate measure of the extracellular spaces appear to remain unchanged on vagal stimulation. Furthermore the extracellular potassium constitutes a very small proportion of the total potassium, so that even a very large increase of the extracellular space could hardly explain the increased influx of K^{42}. A large increase in the ECV would probably also give rise to increased diffusion of other ions from the surroundings (from the blood plasma to the extracellular space). No such effect was observed. It seems therefore plausible that vagal stimulation causes an increased exchange of potassium between the heart and its surroundings (blood plasma) but there is no noteworthy net flux and hence no great changes in concentration in the heart itself. As the slower step is normally between the extra- and intracellular spaces, it is probably this transport which is primarily increased by vagal stimulation.

The mean values for the K^{42} spaces in the vagus stimulated atria and ventricles are considerably greater (approximately 1½ times) than the corresponding mean values for the non-stimulated atria and ventricles after a distribution period of 15–20 minutes. No difference in effect ($P > 0.5$) was observed between the atrium and ventricle.

Vagal stimulation appeared to have no effect on the rate of transport of K^{42} in the sinus venosus, but this may be due to the equilibration having proceeded further in the distribution time allowed. The method used is somewhat rough and the ratio of extracellular to intracellular potassium content is the most unfavourable in the sinus venosus. In other words it cannot be excluded that the same effect does also occur in this part of the heart and this seems quite likely since there is evidence that the principal part of the vagus fibres pass to the pacemaker of the heart (SETEKLEIV 1963). HARRIS and HUTTER (1956) found an increase in both the influx and efflux of K^{42} in isolated sinus venosus from frogs treated with acetylcholine or vagal stimulation.

The Br^{82} content in the heart relative to that in the skeletal muscle does not differ between normal and vagus stimulated toads, but if the Br^{82} bromide space is calculated, this is smaller in vagus stimulated than in normal hearts, as is also

possibly the case for the Na^{24} sodium space. There are several possible explanations for these results. 1) If the radioactive isotopes are not equilibrated with the stable isotopes there may be a decrease in equilibration rate caused by the vagal stimulation. Since presumably the extracellular space is completely or almost completely equilibrated after the distribution period chosen, the difference would in this case be due to a decreased rate of exchange between the extra- and intracellular spaces. Such a difference would, however, need to be rather large to be observed because the intracellular sodium and bromide contents are very small compared with the extracellular so that this explanation seems therefore not very probable but cannot be excluded (*vide infra*). 2) Another explanation may be that both the radioactive sodium and bromide ions were equilibrated with stable isotopes and that the results are due to a net flow of sodium and bromide from the heart either caused by the vagal stimulations as such or by the cardiac arrest produced by the stimulation (e. g. a squeezing out of extracellular fluid into the blood). Such a squeezing effect can, however, probably be excluded since the sulphate space determinations made simultaneously with the Br^{82} bromide and the Na^{24} sodium spaces showed no difference between normal and vagus stimulated hearts.

Some earlier studies (ÖBRINK and ESSEX 1953, KLEIN and HOLLAND 1958, HOLLAND and CERVONI 1962) indicate that the sodium ions also are effected by vagal stimulation. ÖBRINK and ESSEX (1953) for example found that the very high sodium concentration in the sinus node region appeared to decrease during vagal stimulation. HOLLAND and CERVONI (1962) reported that acetylcholine increased the rate of entry of Na^{24} in quiescent or stimulated isolated rabbit atria (equilibrated for 30–40 mins in a Na^{24} solution).

HUTTER (1961) found that acetylcholine or vagal stimulation produced no effect on the bromide ion transport in the frog sinus venosus. It is not easy, however, to observe changes in the flows of sodium or bromide ions between the extra- and intracellular spaces, even if these changes are large, since the intracellular contents of these ions are small.

As mentioned the chloride ions are relatively unimportant

In *chapter IV* data on the distribution of chloride in the heart are reported. A review is also given of earlier methods for the analysis of chloride in biological tissue and of previous investigations on the distribution of chloride in the heart and skeletal muscle. The results of the chloride determinations in different regions of the heart and in the skeletal muscle are given. *The pattern of chloride is similar to that of Na: sinus venosus > atrium > ventricle > sartorius muscle.*

In order to calculate the electrolyte distribution in the extra- and intracellular compartments the size of the extracellular space must be known. Since the chloride space is considered to be a good approximation in many tissues of the extracellular space, this was calculated in different regions of the heart and in the sartorius muscle (the mean values in ml/100 g wet tissue: for sinus 75, for atrium 50, for ventricle 25 and for sartorius 15). *The chloride spaces so calculated were also compared with the corresponding Br^{82} bromide and I^{131} iodide spaces (no differences were found).*

Chapter V describes studies of the distribution spaces for different carbohydrate molecules and sulphate ions, which appear to remain essentially extracellular. The results showed that *the size of the mannitol and sucrose spaces continued to increase greatly after a long distribution period, which was interpreted as indicating entry of these species into the cell.* Maltotriose was less suitable as an extracellular indicator, since the distribution spaces for this substance were extremely large, probably due to its breakdown in the tissue of the toad. *Sulphate seemed to give the best estimate of the extracellular space, at least in the heart, since the sulphate ions very rapidly equilibrated with a volume which probably did not subsequently increase even after a long distribution period* (the mean values for the sulphate spaces in ml/100 g wet tissue were: sinus 63, atrium 49, ventricle 22 and sartorius muscle 17).

In *chapter VI* results of simultaneous Na^{24} sodium, Br^{82} bromide and S^{35} sulphate determinations are compared. *There were no differences between the Na^{24} sodium and Br^{82} bromide spaces in the atrium, ventricle and sartorius muscle, while in*

the sinus venosus the Br^{82} bromide space was larger than the Na^{24} sodium space. In the skeletal muscle the S^{35} sulphate space was smaller than the Na^{24} sodium space, but in the heart there were no significant differences between the S^{35} sulphate spaces and the other ionic spaces determined. The simultaneous determinations made possible more accurate comparisons between different extracellular space approximations and more reliable calculations of the intracellular electrolyte concentrations.

In chapter *VII* the results of the different extracellular space determinations in the different heart regions and the skeletal muscle are discussed. *The sulphate space which seems to be the best extracellular indicator, and other ionic spaces in all heart regions were after short distribution periods larger than the mannitol and sucrose spaces. This may be due to some hindrance in the extracellular equilibration of the latter solutes.*

The extracellular space is different in each region of the heart (sinus venosus > atrium > ventricle) regardless of which substance is used for the ECV determination.

The water content in the heart and sartorius muscle was found to constitute 80–85% of the weight of wet tissue.

The intracellular concentrations of sodium (the estimated mean values varied between –6 and 10 meqv/l calculated from the sulphate spaces) *and potassium* (approx. 100 meqv/l calculated from the sulphate spaces in newly captured toads) *were of the same order of size in all regions of the heart, while the intracellular chloride concentration was not the same* (mean values calculated from the S^{35} sulphate spaces, cf. Table 15, line 1, page 67, expressed in meqv/l: for sinus 24, for atrium 14, for ventricle 5 and for sartorius muscle 3). *Thus for Na and K the differences in total contents in different regions of the heart can be explained entirely by the fact that the extracellular volumes differ, but the variations in chloride content appear to be due partly to the differences in the extracellular volume and partly to intracellular differences.*

This chapter also discusses (a) the relationship between the histological appearance and the spaces determined, (b) factors of importance in the transmembrane transport, such as the

properties of the membrane and those of the solutes the driving forces and the length of the distribution period Also discussed is the Donnan equilibrium between plasma and the extracellular water and its significance in ion distribution

Chapter VIII reviews briefly the theories which seek to explain the ionic asymmetry between the intra and extracellular spaces in excitable tissue the genesis of excitability and possible mechanism of the effect of acetylcholine or vagal stimulation on the heart

Chapter IX describes experiments which showed that *the rate of transport of potassium ions in the heart increases on vagal stimulation while the sulphate ions do not appear to be affected The bromide and possibly also the sodium spaces (determined with radioactive isotopes) appear to be smaller in vagus stimulated than in normal hearts which may be due reduced to a either equilibration rate and/or a net flow of bromide or sodium from the heart* (not so large however that it was observed by the analysis of stable isotopes)

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HELSINKI 1964

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FROM THE DEPARTMENT OF ANATOMY UNIVERSITY OF HELSINKI
SILTAVUORENPENGER, HELSINKI FINLAND

CARBOXYLIC ESTERASES, OXIDATIVE ENZYMES AND CATECHOLAMINES IN THE SUPERIOR CERVICAL GANGLION OF THE RAT AND THE EFFECT OF PRE- AND POST-GANGLIONIC NERVE DIVISION

BY

MATTI HÄRKÖNEN

HELSINKI 1964

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PREFACE

The sympathetic ganglion was proposed as the subject of the present study in 1961 by Professor Olavi Eränkö M D Ph.D Head of the Department of Anatomy University of Helsinki This work is a part of the departmental research project on the histochemistry of the nervous tissue directed by him

I wish to express my deep gratitude to Professor Eränkö for his continued support of my endeavour in the course of the different stages of this study I especially appreciate his never failing readiness to advise me and his valuable criticism

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Mrs Jean M Perttunen, B Sc. corrected the English. Mr Erkki Jarvinen M A carried out the statistical analysis I thank them both for pleasant collaboration and interest in my work.

Finally I owe a great debt of gratitude to my wife for her patient help in the whole course of this study

Helsinki September 1964

Matti Harkonen

CONTENTS

INTRODUCTION

The structure of both the central and the peripheral nervous system includ ing the autonomic ganglia has been studied in detail with the aid of the classical neuro-histological methods (see Stohr 1957) Several studies on the ultrastructure of normal nervous tissue have also been published during recent years (see Palay and Palade 1955 Elfvin 1963a b)

Moreover many new analytical tools have recently become available for the purpose of studying metabolic activities at the cellular level. These new sensitive techniques have in fact also been applied to the study of chemical components of single isolated neurones the work of Hydén and his group has elucidated many of the metabolic relationships between the nerve cells and the surround ing glia (for review see Hyden 1960 1962) Lowry and his associates have suc cessfully used fluorescence micro-analysis to study the enzyme activities of small samples of cortical layers of the brain and even of isolated nerve cells (e g Lowry 1957a b)

Advances in microscopic histochemistry have recently enlarged the scope of this type of investigation Using histochemical methods it is possible for instance to localize enzymatic activity in tissue sections even at the subcellular level Histochemical enzyme methods are also of value in the study of cyto-plasmic organelles (see Novikoff and Essner 1962)

In histochemical systems the relationships of the various tissue components are disturbed as little as possible Therefore the in vivo conditions may be re produced more nearly than in tissue homogenates Many hydrolytic and oxi dative enzymes can now be adequately demonstrated in tissues and cells and valuable information has thus been obtained concerning the correlation of their structure and the metabolic events occurring in them

Somewhat surprisingly relatively few reports are as yet available on the histo-chemistry of nervous structures The autonomic nervous system has been espe cially neglected except for cholinesterases whose histochemical demonstration in the sympathetic system provided valuable new observations leading to a new hypothesis of neurohumoral transmission (Koelle 1962)

The present study was designed to fill in some of the obvious gaps in our knowledge of the enzyme histochemistry of the sympathetic nervous system by an investigation of the distribution and activity of cholinesterases non specific carboxylic esterases and oxidative enzymes in the superior cervical ganglion of the rat under normal conditions and at varying periods after pre or postganglionic denervation The distributions of different types of esterases have also been compared with those of dehydrogenases amine oxidase and

catecholamines Some of the enzyme activities have been determined quanti tatively in order to study the effect of denervation Before these enzyme studies are described some observations are reported on the electron microscopic structure of the sympathetic ganglion

GENERAL MATERIAL AND METHODS

Male rats of the Sprague Dawley strain were used as experimental animals Their ages ranged from 3 to 12 months The total number of rats examined was well over 500 The animals were killed by decapitation under light ether anaesthesia Both superior cervical ganglia were removed immediately thereafter

For histochemical studies formalin fixed fresh frozen or freeze dried ganglia were used Formalin fixation was carried out overnight at +4 C with formol-calcium (1 volume 35 formaldehyde neutralized over calcium carbonate 3 volumes of distilled water and 6 volumes of 2 calcium chloride) or formol Macrodex (Planteydt 1961) After fixation sections were cut at 10 μ with a freezing microtome. They were stained free floating The freeze-drying and embedding procedures are described in connexion with the catecholamines (p 67) The sections were deparaffinized in light petroleum ether and hydrated in graded acetone water solutions (Burstone 1957b)

For the demonstration of formalin resistant oxidative enzymes minute pieces of ganglia were fixed in graded concentrations (0 7 1.3 and 2 0) of formaldehyde in chilled (+4 C) Hanks balanced salt solution (BSS) for 6 to 20 minutes (Walker and Seligman 1963) Thereafter the tissue blocks were treated in the same way as the fresh ganglia.

For the preparation of fresh sections the ganglia were covered with 10 / gelatin and frozen with solid carbon dioxide or liquid air When the effect of denervation was studied the ganglia were first removed from the animals and stored in small tightly closed glass tubes in ice (for 15—20 minutes) They were then mounted together on a metal holder covered with gelatin and frozen In this way 10—15 ganglia could be attached on the same tissue holder and subsequently cut at one stroke. This guaranteed that all the sections were equally thick and were treated on the same coverslip in an identical manner This is important when the effect of experimental conditions is being studied

The tissues were sectioned with a cryostat microtome at 6—10 μ. The sections were then mounted on coverslips and dried at room temperature for 5—10 minutes Some cryostat sections were postfixed in formol Macrodex or in acetone at +4 C for 15 minutes.

For quantitative determinations of enzyme activity the ganglion was removed and weighed in a tightly closed Potter Elvehjem micro-homogenizer and homogenized in 200 μl of cold distilled water under constant cooling with ice water

For glucose 6-phosphate dehydrogenase determination 80 μl of the homogenate was centrifuged for 15 minutes at 3000 × g at +4 C, and the supernatant used for the assay Thirty μl of water was added to 60 μl of homogenate and this was used for determination of esterases The remaining homogenate was diluted with an equal volume of water and 50 μl of this mixture was used for the determination of NADH-cytochrome c reductase activity and protein content A further portion of the mixture (ca 30—40 μl) was again diluted with an equal volume of water and this mixture was used for measurement of lactate and malate dehydro-

genase activities The tubes were stored in ice until use The oxidative enzyme determinations were carried out on the same day and the esterase activities and protein content were measured on the following day

For starch gel electrophoresis the supernatant was prepared as follows The ganglia were homogenized with distilled water in a small glass homogeni er (2—4 mg of fresh tissue/10 μl water) The homogenate was then frozen and thawed 5 times and centrifuged for 30 minutes at 3500 × g or 25000 × g at +4 C In denervation experiments two ganglia were usually pooled

Denervation was performed under ether anaesthesia with the aid of a dissecting microscope The superior cervical ganglion was denervated in three ways 1) preganglionically by dividing the cervical sympathetic trunk approximately 5 mm below the ganglion 2) post ganglionically by cutting all visible postganglionic nerve fibres 3) both preganglionically and postganglionically The success of the operation and the occurrence of functional recovery could be followed by examination of the subsequent narrowing of the palpebral fissure and constriction of the pupil (Horner's syndrome) The postoperative recovery period was varied from twelve hours to 150 days In every experiment the contralateral unoperated ganglion was used as a control

I A BRIEF ACCOUNT OF THE ULTRASTRUCTURE OF SYMPATHETIC GANGLION CELLS

EARLIER INVESTIGATIONS

The sympathetic ganglion has been used in a number of studies in which the ultrastructure of the nerve cell has been the subject of inquiry Palay and Palade (1955) first described the structure of the nerve cell in detail. Their material consisted of sympathetic spinal and intramural ganglia of the gut as well as cells of the cerebellar cortex, which made it possible to compare the characteristics of different types of nerve cells Further information about the normal electron microscopic structure of the sympathetic ganglion has been provided by Hess (1958) Smith (1959) and Elfvin (1963a b)

Only a few electron microscopic observations have been made on any nerve cells undergoing the axon reaction. Hartmann (1954) confirmed his earlier light microscopic finding (1948) that following division of the axon the number of mitochondria is increased in the perikaryon of the motor nerve cell. Later Hudson, Lazarow and Hartmann (1961) quantitatively investigated the changes in the number and volume of mitochondria during the axon reaction. Vesiculation and disintegration of mitochondria were observed by Causey and Hoffmann (1955) in the spinal ganglion of the rat and by Barton and Causey (1958) in the sympathetic ganglion of the rabbit. Smith (1961) on the contrary reported that the mitochondria in the dorsal root ganglion hypertrophied after denervation Andres (1961) described the structural alterations of mitochondria which occur at late stages of chromatolysis.

In all the above mentioned studies methacrylate was used as embedding medium. Therefore it seemed worthwhile to re examine the axon reaction in the sympathetic ganglion with newer techniques allowing better preservation of the cell organelles

METHODS

Small pieces of fresh ganglia were fixed in 1 % osmium tetroxide in 4 5 % sucrose buffered to pH 7 4 (Palade 1952) or in the solution prescribed by *Rhodin (1954) and Zetterqvist (1956)* for 2 hours at +4 C. They were then dehydrated in alcohol series and embedded in methacrylate (methyl- and butylmethacrylate 1 9 with 1 % benzoyl peroxide as catalyst) or in Epon 812 (Shell) (Luft 1961) Sections were cut at approximately 800 Å with a LKB ultrotome fitted with glass knives The sections from Epon-embedded material were poststained with 1 % lead citrate for 5 minutes (Reynolds 1963)

The electron microscopes used were the Akashi TRS-50 and Siemens Elmiskope I

RESULTS

NORMAL GANGLION

The nerve cells of the superior cervical ganglion and the nuclei in them were as is well known usually round The small Nissl bodies appeared as aggregates of parallel membranes surrounded by a large number of ribosomes There were also free ribosomes in the cytoplasm Typical Golgi complexes with small vacuoles and tubular vesicles were sometimes seen in the perinuclear area The cytoplasm contained numerous mitochondria of variable size and shape They were as a rule evenly distributed throughout the cytoplasm However a ring formed by mitochondria was regularly seen immediately around the nucleus (Fig 1) The typical cristae mitochondriales were numerous running perpendicular to the long axis of the mitochondrion (Fig 3)

In addition to the mitochondria the cytoplasm of the nerve cells contained dense bodies which were round or oval and somewhat larger than the mitochondria These bodies presumably lysosomes were not confined to any particular region of the cytoplasm They were homogeneous or granular and sometimes a limiting membrane was observed and signs of internal organization in the form of cristae

The nerve cells were surrounded by satellite cells These contained ellipsoid nuclei which were smaller and denser than the nuclei of the neurones The mitochondria of the satellite cells were slightly larger and darker than those of the neurones

POSTGANGLIONIC DENERVATION

Nuclear changes were visible within 24 hours after the operation The nuclei adopted a more eccentric position and the structure of the nucleoplasm became disorganized Later the nuclear membrane became convoluted and finger like projections were extruded into the cytoplasm

Denervation caused reduction and progressive disintegration of the ergastoplasmic reticulum in the central region of the perikaryon with peripheral condensation of the degenerating reticulum Four weeks after the operation there was evidence of reconstitution of ergastoplasmic aggregates in the cytoplasm

The mitochondria already showed some swelling on the first postoperative day but the cristae remained intact The volume of individual mitochondria increased progressively however reaching a maximum between the 7th and 15th days The swelling was very marked at this time and at low power the mitochondria looked like empty vesicles (Fig 2) However at higher magnification remnants of mitochondrial cristae could be recognized (Fig 4) In material embedded in methacrylate the inner structure clearly visible in sections of specimens embedded in Epon was entirely lost but invaginations of the outer mitochondrial membranes could be seen in many instances The swollen mitochondria were usually located in the central region of the nerve cell cytoplasm where the Nissl substance had disappeared The numbers of mitochondria and lysosomes in some normal and chromatolytic cells were calculated but no significant differences were found



Fig 1 Electron micrograph of a normal sympathetic ganglion cell Note the ring formed by an accumulation of mitochondria around the nucleus x 2500

Fig 2 Electron micrograph of a chromatolytic sympathetic ganglion cell 10 days after operation The nucleus has taken an eccentric position and the nuclear membrane is somewhat convoluted The ergastoplasmic aggregates have disappeared in the central region of the pericaryon Note the swollen mitochondria. x 2500

Fig 3 The normal ganglion cell at a higher magnification Numerous ribosomes are seen both free and lining the ergastoplasmic tubules All the mitochondria are similar x 7500

Fig 4 The same ganglion cell as in Fig 2 In almost all swollen mitochondria remnants of mitochondrial cristae can be recognized x 7500

The mitochondria in the satellite cells or in neurones not subjected to axotomy exhibited a normal structure. This shows that the mitochondrial swelling of the chromatolytic neurones was not an artifact due to fixation or embedding.

On the fourth week after division of the nerves less mitochondrial swelling was observed. Two or three months after axotomy the ultrastructure of most of the ganglion cells was normal again.

DISCUSSION

The observations of the present study are in agreement with the majority of previous studies concerning mitochondrial swelling during the axon reaction (Causey and Hoffmann 1955, Barton and Causey 1958, Hudson et al. 1961). The degree of mitochondrial swelling seems to depend on the type of nerve cell. However, it was first shown in the present study that when Epon was used as embedding medium the changes were not so extensive as and the mitochondrial structure was better preserved than in specimens embedded in methacrylate. This indicates that methacrylate embedding has an intensifying effect on the swelling of already damaged mitochondria. The increase in the number of mitochondria which has been reported to occur in motor nerve cells (Hartmann 1948, 1954 and Hudson et al. 1961) and in spinal ganglion cells (Smith 1961) during chromatolysis could not be detected in the sympathetic ganglion cells.

II CARBOXYLIC ESTERASES IN THE SUPERIOR CERVICAL GANGLION

GENERAL FEATURES OF CARBOXYLIC ESTERASES

Carboxylic esterases hydrolyse the carboxylic acid esters of alcohols phenols and naphthols. Some of these enzymes are also known to break amide bonds (Myers et al 1957 Hoosu and Glenner 1964a b) On the other hand certain proteolytic enzymes e g trypsin chymotrypsin and cathepsin are capable of hydrolysing ester linkages (Dixon and Webb 1958) Biochemical data show that esterases differ from each other with respect to substrate preference and in their behaviour towards various activators and inhibitors (Aldridge 1954) Further different tissues and animal species evidently have strikingly different types of esterase activities (Augustinsson 1948)

Histochemically esterases have been divided into two main groups non specific esterases (ns.Es) and cholinesterases (ChEs) (e.g Pearse 1960) The dividing line between non specific esterases and lipases is to some extent arbitrary Broadly speaking the esters of short-chain fatty acids (C 2-C 4) are the substrates for non specific esterases and the long-chain esters (C 8 and upwards) for lipases

In most older works it was customary to use different substrates for the characterization of esterases Aldridge (1953a b 1954) however pointed out that it is more useful to employ a variety of activators and inhibitors than various substrates With the aid of diethyl p-nitrophenyl phosphate (E600) he was able to distinguish two basic types of carboxylic esterases A-esterase which is not inhibited by concentration up to 10^{-3} M of E600 and which hydrolyses acetate esters more rapidly than the corresponding butyrates and B-esterase which is inhibited by E600 at a concentration of 10^{-8} M and splits butyrates at a rate equal to or faster than acetates Lipase type esterases have been considered to belong to the group of B-esterases (Myers et al 1955) The third type of enzyme C-esterase which also hydrolyses simple carboxylic esters, is resistant to organophosphorus compounds but does not hydrolyse them like A-esterase (Bergmann et al. 1957 Bergmann and Rimon 1958) It also differs from A-esterase in the respect that it is activated by a low concentration of p-chloromercuribenzoate (PCMB)

A distinction can be drawn between ns Es and ChEs according to their susceptibility to eserine. The former enzymes are but little affected even by 10^{-3} M eserine while ChEs are completely inhibited by a 10^{-6} M solution of this compound (Easson and Stedman 1937 Richter and Croft 1942) Unfortunately there is no selective method of inhibiting the ns Es without affecting the ChEs

Two types of ChEs are recognised one is inhibited by high concentration of acetylcholine while the other is not (Alles and Hawes 1940) The first is called »true» or acetylcholinesterase (AChE) and the the second »non specific or pseudocholinesterase (ns ChE) (Mendel and

Rudney 1943 Augustinsson and Nachmansohn 1949) In biochemical studies acetyl β-methyl choline has proved a specific substrate for AChE and benzoylcholine for ns ChE (Mendel et al. 1943 Mendel and Rudney 1943) Moreover ns ChE splits long-chain choline esters (e g butyrylcholine) more rapidly than AChE does (Ord and Thompson 1952) The use of these specific substrates for both ChEs has made it possible to study selective cholinesterase inhibitors and their inhibitory power (Aldridge 1953a Denz 1954 Bayliss and Todrick 1956 Holmstedt 1957a) In these studies the most selective inhibitors of AChE turned out to be 1 5-bis-(4-trimethylammoniumphenyl) pentan 3-one-diiodide (62C47) and 1 5-bis-(4-allyl dimethylammoniumphenyl) pentan 3-one-diiodide (284C51) while tetra isopropylpyrophosphoramide (iso-OMPA) and bis-monoisopropylamido-phosphoryl fluoride (Mipafox) were potent inhibitors of ns ChE.

HISTOCHEMICAL DEMONSTRATION OF CARBOXYLIC ESTERASES

The first histochemical method for the demonstration of *cholinesterases* was developed by Gomori (1948) He used long-chain fatty acid esters of choline as substrates (e g myristoyl choline) and fixed, paraffin-embedded tissues Evidently it is possible to demonstrate only ns ChE by this method (Koelle and Friedenwald 1949)

In a more widely employed method thiocholine esters serve as substrates instead of choline (Koelle and Friedenwald 1949 Koelle 1950 1951) Several modifications of the thiocholine technique have been reported, e g by Gomori (1952a) Coupland and Holmes (1958) and Holmstedt (1957b)

Indoxyl and azo-coupling methods originally developed for demonstration of total esterase activity can also be used for the demonstration of ChEs alone The acetates are hydrolysed in both techniques by ChEs (Holt 1952 Underhay 1957) Using specific inhibitors the ChEs and ns Es can be characterized (Pepler and Pearse 1957 Allen et al. 1957 Eranko 1959)

Histochemical methods for demonstrating *non specific esterases* are based on the use of a substrate hydrolysed by as many carboxylic esterases as possible and on inhibition of ChEs by eserine. These esterases may further be characterized by using E600 as inhibitor Esterase activity resistant to E600 have been suggested to be due to peptidases (Pepler and Pearse 1957)

In the simultaneous azo-coupling methods for esterases first presented by Nachlas and Seligman (1949a) the substrate was β-naphthyl acetate Enzymically liberated β-naphthol was captured by diazotized α-naphthylamine α Naphthyl acetate was introduced by Gomori (1952a b) He was also the first to use naphthol AS acetate (Gomori 1952b) Pearse (1953) Burstone (1957a b) and Gossner (1958) further improved the precision of esterase localization by introducing the substituted naphthols

A new principle for the demonstration of esterases was independently suggested by Barrnett and Seligman (1951) and by Holt (1952 1958) It was based on the production of indigo from different indoxyl esters.

DISTRIBUTION OF CARBOXYLIC ESTERASES IN THE NERVOUS TISSUE

Cholinesterases

The ChE activity of the sympathetic ganglia has been the subject of several studies since Glick (1937) found that both AChE and ns ChE are present in the superior cervical ganglion and may be situated at the synapses.

The first paper on the distribution of ChE in the peripheral nervous tissue was published by Koelle (1951) He observed that in the stellate ganglion of the cat AChE activity was limited to the entering preganglionic axons and their terminations as well as to a few ganglion cells and nerve fibres starting from them. In the superior cervical ganglion of the cat the picture was similar except that the intensely stained ganglion cells were far less numerous (Koelle 1951 1955) The pattern of staining has been observed to be somewhat different in the sympathetic ganglia of the rabbit the rhesus monkey (Koelle 1955) the rat (Koelle 1954) and several other species including man (Cauna et al. 1961) However in all these species all the neurones were lightly or moderately stained. Gerebtzoff (1959) emphasized that the synaptic localization of AChE was especially clear in the dog The neurones of the Auerbach plexus of all the species examined also contained ns ChE, which in other ganglia was noted only in the glial cells except for occasional neurones of the superior cervical ganglion of the rat (Koelle 1951 1954)

Fredriksson and Sjoqvist (1962) and Sjoqvist (1962) used Holmstedt s modification of Koelle s thiocholine method and found some activity even in those sympathetic ganglion cells of the cat which Koelle (1951) had stated to be inactive The claim that there are nerve cells without AChE activity has been criticized from the biochemical point of view by Nachmansohn (1960) who has demonstrated AChE in most nervous tissues irrespective of their function

Giacobini (1959) estimated the AChE activity of single nerve cells Using a microgasometric technique he was able to show that AChE was present in most sympathetic cells of the rat and the frog In both animals however no activity could be demonstrated in a certain number of cells even after prolonged incubation times In some cells ns.ChE was also present

Parasympathetic ganglion cells have been found to exhibit an intense AChE activity (Koelle 1951 1955 Fukuda and Koelle 1959) However some authors have claimed that it is localized mainly in the synaptic membranes (Szentagothai et al. 1955) Preganglionic parasympathetic fibres have been found to exhibit only slight AChE activity while postganglionic fibres show intense staining all the way to the periphery Ns ChE is again limited to the capsular cells and Schwann cells (Koelle 1951 1955)

Since the known cholinergic neurones (preganglionic sympathetic fibres parasympathetic and motor neurones) have been found to possess high levels of AChE, Koelle (1951 1955 1962) and Sjoqvist (1962) suggested that the heavily stained neurones in the sympathetic ganglia might also be cholinergic in nature.

All the sensory neurones in the spinal ganglia the nodose ganglion and the trigeminal ganglion of the cat the rabbit the rhesus monkey (Koelle 1951 19 5) and the guinea pig (Cauna and Naik 1963) contain a small amount of AChE. Tewari and Bourne (1962a) on the other hand, claimed that some cells in the spinal ganglia of the rat are without AChE activity and only a few neurones turned out to be positive in the trigeminal ganglia (Tewari and Bourne 1963b c)

Non specific Esterases

Using acetone fixed and paraffin-embedded tissues and β-naphthyl acetate as substrate Nachlas and Seligman (1949a) were unable to detect any esterase activity in the brain or spinal cord of some mammals but an intense esterase activity was seen in the coeliac ganglion cells of the rat. The authors later concluded that only aliesterases (= ns Es) were demonstrated by their technique (1949b) Using the same technique other workers observed an intense reaction in and around the motor nerve cells of the spinal cord as well as in the spinal and sympathetic ganglion cells (Hard and Fox 1951 Sávay et al 1953)

Chessick (1953) and Gomori and Chessick (1953) studied esterases in the central nervous system of some mammals using α naphthyl acetate and naphthol AS acetate as substrates They employed specimens embedded in paraffin after fixation in cold formalin In dog tissues the α-naphthyl method stained fibre tracts while the naphthol AS method demonstrated the nerve cells. In the rat brain both methods revealed predominantly cellular activity and the glial reaction was absent or minimal. »Alpha-esterase» was completely inhibited by 10^{-4} M DFP and almost completely by the same concentration of eserine and TEPP »AS-esterase» appeared to be insensitive to all three inhibitors Malaty and Bourne (1955) used the same substrates and found hardly any »alpha-esterase» in brain tissue whereas »AS-esterase» was abundant especially in the Purkinje and pyramidal cells Eserine had no effect on »AS-esterase» In the nerve cells esterases hydrolysing naphthol AS acetate have been observed to coincide with the sites of lipoid pigment (Gomori 1955 Gedigk and Bontke 1956)

In the spinal cord the neuronal esterase splitting β-naphthyl acetate has been reported to be AChE (Ravin et al. 1953)

When fresh frozen sections and indoxyl acetate or butyrate were used for the localization of esterases moderate activity was found in the neurones of the central nervous system (Barrnett and Seligman 1951 Barrnett 1952) The reaction obtained with indoxyl acetate was to some extent inhibited by eserine but that with butyrate was not Eserine had no effect on an intense perinuclear enzyme activity seen in most cells of the brain cortex with 5-bromoindoxyl acetate (Pearson and Grose 1959) With the same substrate Thomas (1963) found a variably positive esterase reaction in human spinal ganglion cells

The esterases in the neurosecretory centres and their functional significance have been studied by Pearse (1955) Pepler and Pearse (1957) and Arvy (1961) The former authors employing various substrates and inhibitors were able to identify six types of enzymes with different distributions

Tewari and Bourne studied the spinal (1963a) and trigeminal ganglia (1963b c) of the rat. The reaction obtained with naphthol AS D acetate in the trigeminal ganglion was positive in all the cells but in the spinal ganglion some neurones were inactive In supposedly aging nerve cells an increased esterase activity was observed (Bourne 1957)

Eränkö et al. (1962 b) observed that the ganglion cells of the adrenal medulla exhibit both types of ChE activity as well as both E600-sensitive and E600-resistant ns E activity

Effect of Denervation on Esterase Activity

Division of the preganglionic trunk results in a marked decrease of total ChE activity of the sympathetic ganglion (Brucke 1937) Sawyer and Hollinshead (1945) observed that the AChE activity fell to 25 and that of ns ChE to 75 % of the original level. This was confirmed by Hard et al. (1951)

By histochemical methods it was found that AChE completely disappeared from the majority of nerve fibres but remained intact in the nerve cells the ns ChE picture was not affected (Koelle 1950 1955 Brown 1958 Snell 1958 Taxi 1961 Fredricsson and Sjoqvist 1962, Holmstedt et al. 1963) After division of the splanchnic nerve the adrenal medullary ganglion cells retained their strong AChE activity although no activity remained in the preganglionic nerve net (Eranko et al. 1959 1962a) Parasympathetic ganglia behave in the same way after pre ganglionic denervation (Szentagothai et al. 1955)

Manometric estimations of the total ChE activity in the superior cervical ganglion of the rat have shown that about 50 % of the activity was lost after sectioning the postganglionic nerves this phenomenon was accompanied by failure of ganglionic transmission, while the acetylcholine output to the blood was still normal (Brown et al. 1952, Brown and Pascoe 1954 McLennan 1954 Dhar 1958) These results were confirmed histochemically by Brown (1958) who showed that axotomy may affect AChE not only in the cell bodies but in the preganglionic fibres as well. Taxi (1961) found a similar disappearance of the enzyme from the nerve cells but the interstitial activity rather increased Fredricsson and Sjoqvist (1962) and Sjoqvist (1962) on the other hand concluded that axotomy induced a decrease of AChE in sympathetic neu rones but that the loss of activity was seldom total.

Gerebtzoff and Vandersmissen (1956) observed a decrease in the ChE activity of the dorsal vagal ganglion 15 days after cutting the vagus The synaptic terminals around the ganglion cells remained active The motoneurones also exhibited a definite decrease in AChE activity during axon reaction (Schwarzacher 1958 Chacko and Cerf 1960)

Division of the splanchnic nerve did not cause any changes in ns E activity in the adrenal medullary ganglion cells (Eranko et al. 1959 1962a) There are no reports concerning the ns E activity during chromatolysis

STARCH GEL ELECTROPHORESIS OF CARBOXYLIC ESTERASES

Hunter and Markert (1957) were the first to report a method for the separation of esterases by zone electrophoresis in starch gel In a subsequent series of papers Hunter and his collaborators have characterized esterases in several species and tissues with different substrates and inhibitors (Allen et al 1957 1958 Markert and Hunter 1959 Allen and Hunter 1960 Hunter and Burstone 1960 Hunter and Strachan 1961) Esterases were thought to comprise a family of distinct enzymes with overlapping specificities The differences in zymograms obtained with different substrates were apparently in most instances due to differing rates of reaction and not to absolute substrate specificity

When adrenal medullary esterases were studied by histochemical and starch electrophoretic techniques it was found that eserine inhibited the majority of esterases in sections but had hardly any effect on electrophoretically mobile esterases (Allen et al 1958)

The esterases of the adrenal medulla, the adrenal cortex and the brain of the rat and the hamster were characterized by Eranko et al (1962 c d) In all of these organs several reacting bands were observed and these differed from each other with regard to substrate and in hibitor characteristics When fresh or formalin fixed sections were subjected to electrophoresis most of the esterase activity remained in the section (Eranko et al. 1962b) It was concluded that the esterases histochemically demonstrable in tissue sections are not the same as the enzymes which are seen in starch zymograms Markert and Hunter (1959) indeed showed that only 50 of the total esterase activity was electrophoretically mobile

Barron et al (1961) used six different substrates for studying esterases in human and rat brains with vertical electrophoresis obtaining several bands

In several studies an AChE band has been demonstrated with acetylthiocholine and α naphthyl acetate near the origin (Eranko et al. 1962 c d Barron et al 1961 1963) Lawrence et al. (1960) also noticed that ChE usually occurred in fractions with low mobility

METHODS

Preparation of Tissues

Preparative procedures have been described in detail under *General Material and Methods* Unfixed and fixed cryostat sections frozen sections prepared from formalin fixed ganglia and sections cut from freeze-dried paraffin-embedded ganglia were used The section thickness was 6—10μ.

Histochemical Methods

Techniques Employed with Different Substrates

Thiocholine Esters

Gomori s (1952a) modification of Koelle s (1951) thiocholine method was employed for the demonstration of cholinesterases Twenty milligrammes of acetylthiocholine iodide or butyryl thiocholine iodide diluted in 2—3 drops of distilled water was added to 10 ml of the stock solution The incubation times in the substrate solutions were 1—3 hours

After incubation the sections were rinsed in sodium sulphate saturated at 37 C and immersed for one minute in 1 % ammonium sulphide solution saturated with copper sulphide (Palkama 1961) Finally the sections were washed in distilled water and mounted in glycerol jelly

α Naphthyl Esters

The technique was essentially similar to that described by Nachlas and Seligman (1949a) and later modified by Pearse (1960) and by Eranko et al. (1962a)

α Naphthyl acetate propionate butyrate and laurate served as substrates [1] They were stored as 2 % stock solutions made up in acetone The substrate mixture was prepared fresh each time by adding 2.5 ml of either stock solution to 50 ml of 0 1 M phosphate buffer pH 7 4 Blue RR salt (National Aniline Division New York) or sometimes Fast Blue B salt (I C.I Ltd) served as coupling agent The diazonium salt was added dry to the substrate mixture in a concentration of 7 5 mg/ml and mixed by agitation The substrate-coupler mixture was then filtered directly on the fresh frozen sections attached on coverslips and every 2—3 minutes it was replaced by a newly filtered mixture Formalin fixed frozen sections were immersed free floating in the filtered substrate mixture

The optimal incubation time with α naphthyl acetate turned out to be about three minutes with α naphthyl propionate and butyrate it was much longer about 30 minutes Sodium tauro-

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[1] α Naphthyl butyrate was synthesized according to Gomori (1953) using butyrylchloride and α-naphthol in an acetone medium. The α naphthyl acetate and laurate were commercial preparations and the α-naphthyl propionate a gift from Dr R. L. Hunter

cholate was used as an activator for lipases in the naphthyl laurate mixture. Half a millilitre of 10^{-3} M aqueous solution of this substance was added to the incubation mixture to make a final concentration of 10^{-4} M. Sections incubated in solutions without substrates were used as controls. After incubation the sections were rinsed in distilled water, left to stand overnight covered with glycerol to avoid air bubble formation and then mounted in glycerol.

Naphthol AS D Acetate

The technique used has been described by Gossner (1958). Twenty five milligrammes of naphthol AS D acetate (2 acetoxy 3 naphthoic-o-toluidide) was dissolved in 10 ml of dimethylformamide. Fifty millilitres of 0.2 M phosphate buffer pH 6.8 and 100 mg Blue RR salt dissolved in 40 ml of distilled water were added and the mixture filtered on the sections. The best results were obtained using freeze-dried paraffin-embedded specimens or free floating formalin fixed sections. An incubation time of 1—4 hours was used.

Indoxyl Acetates

The method was described by Holt (1958). The substrates were o-acetyl 5-bromoindoxyl and o-acetyl-4-chloro-5-bromoindoxyl. Tris (hydroxymethyl) aminomethane — HCl buffer pH 7.2 and barbital buffer pH 5.0 were used. Sometimes the concentration of the ferro-ferricyanide redox buffer was reduced to 10 % of that prescribed (Barron et al. 1963). The sections attached on the slides or free floating (formalin fixed) were incubated for 4 hours with the former substrate and for 30—60 minutes with the latter. After rinsing in distilled water the sections were mounted in glycerol.

Inhibitor Studies

In order to distinguish between different types of carboxylic esterases the following inhibitors were used.

Eserine salicylate (Merck, Germany) was used at concentrations of 10^{-4} and 10^{-5} M to inhibit the activity of cholinesterases. In order to determine whether the eserine-sensitive component is AChE or ns ChE, iso-OMPA (Light, England) was employed at concentrations of 10^{-3} to 10^{-6} M to inhibit ns ChE and 284C51 or 62C47 (Wellcome Research Laboratories, England) at concentrations of 10^{-5} to 10^{-6} M to inhibit AChE. Organophosphorus resistant esterases were studied with E600 (Bayer, Germany) at concentrations of 10^{-6} M. PCMB at a concentration of 10^{-4} M was used together with E600 to study the possible presence of esterase activity of type C.

The sections were first preincubated for 20—30 minutes in an inhibitor solution in 0.1 M phosphate buffer, pH 7.4, or in the stock solution of the thiocholine method and then transferred to *the ordinary incubation mixture to which the same concentration of the same inhibitor* had been added.

Starch Gel Electrophoresis

The technique used was in principle the same as that of Markert and Hunter (1959) as employed by Eranko et al. (1962c). Measured aliquots (10 µl) of the sample were applied to strips of Whatman No. 1 filter paper (9 × 9 mm) and these were then inserted in a slit cut transversely in a starch block. Two samples were usually inserted in the same starch block at a distance of 8 cm from each other.

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RESULTS

NORMAL GANGLION

Distribution of Esterases in Sections

Reactions Obtained without Inhibitors

Hydrolysis of Thiocholine Esters

Satisfactory results were obtained with fresh frozen cryostat sections and with free floating sections cut from ganglia fixed overnight in cold formalin When *acetylthiocholine* was used as substrate all the ganglion cells were positive in both fresh and formalin fixed material However there was a great variation in the intensity of the reaction in individual cells The interstitial tissue consisting of dendrites nerve fibres and glial elements stained strongly *Butyryl thiocholine* produced a positive reaction only in some ganglion cells and in the interstitial tissue it was not possible to find any esterase positive nerve fibres such as were seen in acetylthiocholine preparations

Hydrolysis of Naphthol Esters

α Naphthyl Acetate — In fresh ganglia all the nerve cells showed a positive reaction (Fig 5) On the basis of the intensity of staining three types of ganglion cells could be distinguished The cells that constituted the largest part of the cell population were moderately active There were a few scattered, very intensely stained cells especially at the caudal end of the ganglion The remaining cells showed intermediate activity between these two cell types The reaction was finely granular in all the cells and the granules showed no definite tendency to concentrate in any part of the cytoplasm

The activity of pre and postganglionic nerve fibres was studied in the long nerve trunks attached to the ganglion All the postganglionic fibres were evenly stained but in the preganglionic trunk there were some fibres which stained less strongly than others In some places the fibres showed a more intense and coarser granular reaction than usual These fibres were refractive in polarized light and they were probably myelinated nerve fibres

The tissue elements usually fibre bundles between and around the ganglion cells were very strongly coloured It was difficult to discriminate amongst the stained elements between axons dendrites and glial cells With an oil immersion objective it was possible to observe in some places that positive structures originated from the perikaryon and branched thereafter

The reaction was essentially similar in fresh cryostat sections and after post fixation in cold formol Macrodex However after postfixation the preservation of the tissue was better and the diffusion artifacts sometimes seen without it were absent In freeze dried preparations the picture was sharpest but otherwise similar to that of the cryostat sections

The sections cut from the formalin fixed ganglia (Fig 6) showed greater differences in the esterase activity of individual ganglion cells than did the fresh sections

α Naphthyl Propionate and Butyrate — α Naphthyl propionate and butyrate (Fig 17) gave similar reactions The activity towards these substrates was much weaker than towards acetate Usually ten times the incubation time was needed to achieve about the same staining intensity as with acetate The tissue components between the ganglion cells then exhibited about as strong an activity as with acetate but the staining of the ganglion cells themselves was weaker A few very active neurones were seen however especially in the caudal end of the ganglion The intracellular location and the activity in the pre and post ganglionic nerve trunks was similar to that seen in the α naphthyl acetate preparations

Formalin fixation (Fig 20) and freeze-drying affected the esterase picture in the same way as when α naphthyl acetate was used as substrate

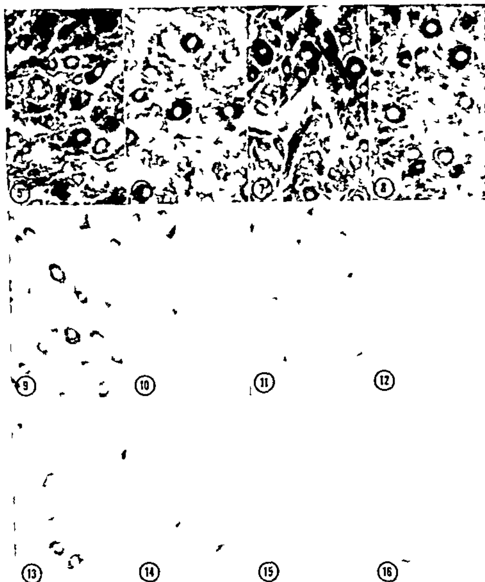
α Naphthyl Laurate — The reaction was entirely negative with or without taurocholate

Naphthol AS D Acetate — When cryostat sections were used the precipitate was coarse and scattered diffusely throughout the ganglion Somewhat better results were obtained using formalin fixed ganglia and free floating sections in which the non specific diffusion was more restricted (Fig 32) Many small and large nerve cells showed only a weak reaction while other cells of the same size were strongly stained The precipitate was still coarse and entirely cytoplasmic In most cells it was restricted to a broad zone around the nucleus in which positive granules were seen In this perinuclear area the number and intensity of the granules varied considerably The interstitial tissue and the pre and postganglionic fibres showed positive granules and were equally reactive and they were less intensely stained than the nerve cells Small highly esterase positive oval »bodies» were observed lining the nerve fibres and around the ganglion cells These cells were not visible in the cryostat sections or freeze dried material. It is probable that these »bodies» are the perikarya of Schwann and capsular cells In the capillaries there were other strongly positive cells which were sausage shaped or wavy in form thus differing clearly from the oval cells It is possible that they were macrophages

When freeze-dried ganglia (Fig 29) were used the reaction product was homogeneous and the different components of the interstitial tissue could be distinguished The activity was quite weak in the ganglion cells despite prolonged incubation for 3—5 hours In many nerve cells strongly esterase positive cytoplasmic granules could be seen perinuclearly

Hydrolysis of Indoxyl Esters

4-Chloro-5 bromoindoxyl Acetate — In fresh and postfixed sections (Fig 23) the reaction product was homogeneous and evenly distributed in faintly stained ganglion cells In some nerve cells the esterase reaction was strong The pre



Figs 5—16 α Naphthyl acetate Blue RR salt. Incubation time 3 min x 175 *Figs 5 7 9 11 13* and *15* Fresh frozen sections *Figs 6 8 10 12 14* and *16* Formol-calcium fixed sections

Figs 5 and *6* No inhibitor
Figs 7 and *8* 284C51 10^{-6} M
Figs 9 and *10* Iso-OMPA 10^{-6} M
Figs 11 and *12* 284C51 10^{-6} + iso-OMPA 10^{-6} M
Figs 13 and *14* Eserine 10^{-6} M
Figs 15 and *16* E600 10^{-6} M

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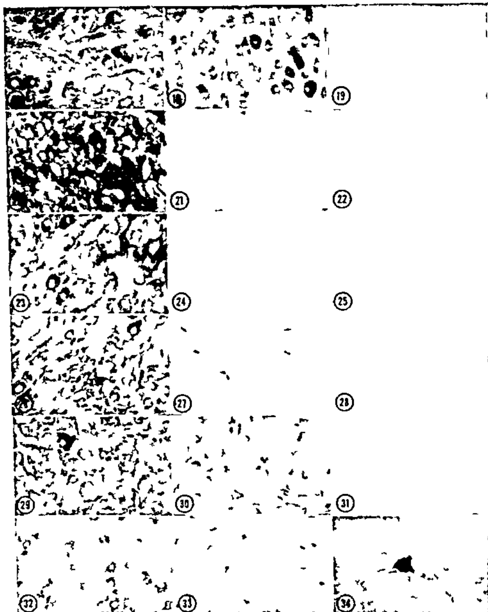
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Figs 17 α Naphthyl butyrate Blue RR salt Incubation time 30 min Fresh frozen section, briefly postfixed in formol Macrodex. No inhibitor x 120 *Fig 18* As *Fig 17* but with 10^{-5} M eserine *Fig 19* As *Fig 17* but with 10^{-5} M E600 *Fig 20* α Naphthyl butyrate formol-calcium fixed. *Fig 21* As *Fig 20* but with 10^{-5} M eserine *Fig 22* As *Fig 20* but with 10^{-5} M E600

Figs 23 4-Chloro-5-bromoindoxyl acetate Incubation time 45 min. Fresh frozen section briefly postfixed in formol Macrodex. No inhibitor x 120 *Fig 24* As *Fig 23* but with 10^{-5} M eserine *Fig 25* As *Fig 23* but with 10^{-5} M E600 *Fig 26* 4-Chloro-5-bromoindoxyl acetate formol-calcium fixed Incubation time 30 min. *Fig 27* As *Fig 26* but with 10^{-5} M eserine *Fig 28* As *Fig 26* but with 10^{-5} M E600

Fig 29 Naphthol AS D acetate Blue RR salt. Incubation time 4 hours Freeze-dried preparation No inhibitor x 120 *Fig 30* As *Fig 29* but with 10^{-5} M 284C51 *Fig 31* As *Fig 29* but with 10^{-5} M iso-OMPA *Fig 32* Naphthol AS D acetate formol-calcium fixed Incubation time 1 hour No inhibitor *Fig 33* As *Fig 32* but with 10^{-5} M E600

result However the hydrolysis of both indoxyl esters and napthol AS D acetate were somewhat inactivated in nerve cells in fresh and freeze dried sections (Fig 30)

In a concentration of 10^{-5} M *eserine* inhibited the reaction in the nerve fibres and glial elements which lost their activity towards all the substrates used in fresh (Figs 13 and 105) and freeze dried preparations The ganglion cells retained a strong activity in spite of eserine but the differences between the individual cells had weakened in sections incubated with naphthyl acetate (Fig 13) propionate or butyrate It was apparent that the neurones with the strongest esterase activity had lost some of their activity and the picture was therefore more uniform than without eserine treatment The reaction was even throughout the cytoplasm in which positive granules were more apparent In postfixed sections the ganglion cells were as active as in fresh sections and in the interstitial tissue some eserine resistant activity could be seen (Fig 18 and Colour Fig 3) In freeze dried ganglia the neuronal activity towards indoxyl and naphthol AS esters was totally absent and with indoxyl acetates only a very faint colour could be seen in fresh and postfixed sections (Fig 24)

In formalin fixed ganglia (Figs 14 21 and 27) there was some eserine resistant activity left in the interstitial tissue but the actual nerve cells had lost much of their activity In some cells a clear perinuclear activity was still observed When the ganglia were fixed in formalin solution containing 20 per cent sodium acetate (Hannibal and Nachlas 1959) activity towards the same naphthol esters was much less inactivated than in formol-calcium fixed sections However the cells shrank and the morphological picture was therefore not satisfactory The addition of »Methocel« (methyl cellulose) (Hannibal and Nachlas 1959) did not prevent the inactivation of esterase by formalin Eserine had hardly any effect on naphthol AS esterase activity but activity towards indoxyl esters (Fig 27) was almost totally inhibited except in the small oval cells the macrophages and in some ganglion cells in which perinuclear indigo granules could be seen

Iso OMPA (10^{-5} M) had an effect almost similar to that of eserine with all the substrates used in fresh (Fig 9) postfixed freeze dried (Fig 31) and formalin fixed ganglia (Fig 10) The ganglion cells however seemed to exhibit a little more activity than those in eserine inhibited sections

The effect of the combination of *iso OMPA* (10^{-5} M) and *284C51* (10^{-5} M) (Figs 11 and 12) was similar to that of iso-OMPA alone except that the neuronal activity was somewhat weaker especially when indoxyl acetates were employed

In a concentration of 10^{-5} M *E600* inhibited all esterase activity in fresh or postfixed cryostat sections (Figs 15 19 and 25) and in freeze dried preparations demonstrable irrespective of the substrate used

In formalin fixed ganglia the esterase activity demonstrable with α naphthyl acetate (Fig 16) propionate or butyrate (Fig 22) was also strongly inhibited However in the interstitial tissue some small oval esterase positive cells were seen (Fig 34) and positive perinuclear granules were observed in a few ganglion cells In indoxyl (Fig 28) and naphthol AS esterase (Fig 33) preparations the number of these oval cells was much larger than in sections incubated with naphthyl esters The naphthol AS esterase activity of the ganglion cells also

turned out to be partly resistant to the action of E600 (Fig 33) The remaining cytoplasmic activity was mainly perinuclear The ganglion cells in sections incubated with either 5 bromo- or 4-chloro-5 bromoindoxyl acetate showed a few perinuclearly situated granules especially with prolonged incubation times (Colour Fig 5)

Distribution of Different Types of Esterases in Sections

In the following description activity resistant to eserine but sensitive to E600 is referred to simply as E600-sensitive non specific esterase (E s ns E) activity and activity resistant to both these inhibitors as E600 resistant non specific esterase (E r ns E) activity even though it is realized that the latter type of activity may be due to peptidases such as cathepsin (Pepler and Pearse 1957)

The distribution and respective contributions of AChE and ns ChE to the total esterase activity were studied acetylthiocholine and iso-OMPA being used to demonstrate AChE selectively and butyrylthiocholine and 284C51 for ns ChE.

Acetylcholinesterase

All the ganglion cells in the superior cervical ganglion were positive and could be divided into two groups on the basis of their AChE activity (Fig 36) There were a few very intensely stained nerve cells especially in the caudal part of the ganglion Most of the cells showed moderate activity however although some variation occurred among these cells too In fresh sections the staining extended throughout the cytoplasm whereas the nuclei remained blank Most of the preganglionic fibres showed intense activity The ganglion cells were surrounded by similarly stained ramifying fibres which terminated as expanded bulbs on the surface of the ganglion cells The postganglionic fibres were slightly stained, but some single fibres with higher activity were occasionally seen

In formalin fixed sections (Colour Fig 1) the preganglionic fibres and terminals seemed to come out better The activity inside the ganglion cells was no longer homogeneous but irregular and the distribution pattern suggested that the activity might be concentrated at ergastoplasmic membranes

Among the other substrates used only indoxyl acetates seemed to be hydrolysed by AChE because 284C51 had a slight inhibitory effect on this reaction in nerve cells

Non specific Cholinesterase

Ns ChE was mainly localized in tissue elements surrounding the neurones (Fig 42 and Colour Fig 2) Similar and uniform enzyme activity was also observed in the post and preganglionic nerve bundles being probably situated in the Schwann cells covering the axons However a few neurones exhibited considerable or moderate ns ChE activity in the cytoplasm The majority of the cells were completely negative Most of the activity with α naphthyl and especially with indoxyl esters was due to ns ChE since it could be abolished

with either eserine or iso OMPA The strongly esterase positive ganglion cells visualized with the aid of these substrates probably contained ns ChE because the inhibitors used eliminated the differences between individual cells Naphthol AS D acetate demonstrated ChE activity only in freeze dried ganglia

E600 sensitive Non specific Esterase

This enzyme was well demonstrated using α naphthyl acetate as substrate (Fig 48 and Colour Fig 3) Indoxyl acetates and naphthol AS D acetate were clearly less satisfactory substrates especially the former

A strong formalin sensitive E s ns E activity was observed in the nerve cell bodies while intercellular structures showed only a weak soluble esterase activity

E600 resistant Non specific Esterase

This enzyme is very soluble and could be demonstrated only after immobilization by formalin fixation Naphthol AS D acetate demonstrates this type of esterase activity mainly (Fig 33) With 4-chloro 5 bromoindoxyl acetate the reaction in the ganglion cells was very weak but when the incubation time was prolonged to two hours small perinuclearly situated granules could be seen in most nerve cells (Colour Fig 4) The small oval cells (capsular cells Schwann cells and macrophages?) exhibited very strong activity with both substrates

Starch Gel Electrophoresis

Reactions Obtained without Inhibitors

Hydrolysis of Thiocholine Esters

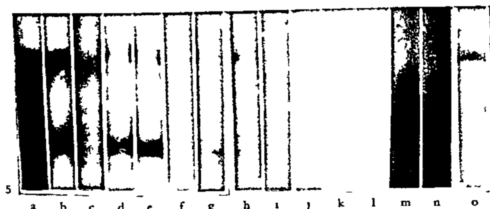
When *acetylthiocholine* or *acetylthiocholine* and *butyrylthiocholine* (Fig 35) together were used for demonstration of cholinesterases three sharp anodal bands (C1 C2 and C3) and a broad zone (C4) between and overlapping C2 and C3 could be seen *Butyrylthiocholine* alone produced the broad zone C4

Hydrolysis of Naphthol Esters

α Naphthyl Acetate — The largest number of reactive bands was observed when α naphthyl acetate was used as substrate (Fig 35 and Colour Fig 5) A strongly positive enzyme reaction was obtained not only in the starch slab but also at the origin of the starch block and on the filter paper strip to which the enzyme extract was originally applied In the starch slab altogether 11 positive zones were obtained and they were designated 1—11 A broad diffuse zone covering the bands 4 and 5 in the middle of the block proved to be a special fraction and was called band 6 Band 10 was not always seen but when the tissue extract was more concentrated it appeared as a weak narrow band

α Naphthyl Propionate and Butyrate — The zymograms obtained with these two substrates were essentially similar They differed from acetate zymograms

1 2 3 4



Colour Fig 1 AChE activity in formalin fixed ganglion x 250

Colour Fig 2 Ns ChE activity in formalin fixed ganglion. x 250

Colour Fig 3 E s ns E activity in formol Macrodex postfixed section α Naphthyl acetate x 250

Colour Fig 4 E r ns E activity in formalin fixed ganglion. 4-Chloro-5 bromoindoxyl acetate incubation prolonged to 2 hours x 250

Colour Fig 5 Esterase zymograms obtained after simultaneous electrophoretic separation. a, α naphthyl acetate b α naphthyl acetate + eserine 10^{-5}M c α naphthyl acetate + E600 10^{-5}M d α naphthyl butyrate e α naphthyl butyrate + eserine 10^{-5}M f α naphthyl butyrate + E600 10^{-5}M g naphthol AS D acetate h naphthol AS D acetate + eserine 10^{-5}M i naphthol AS D acetate + E600 10^{-5}M j 4-chloro-5-bromoind acet. k, 4-chloro-5-bromoind acet + eserine 10^{-5}M l 4-chloro-5-bromoind acet + E600 10^{-5}M, m, acet thiochol. +

only in so far that some bands (5 6 7 8 10) obtained with acetate were lacking while the others had the same location (Fig 35 and Colour Fig 5) However variations in the intensity of the reaction in various bands were noticed with the different substrates In acetate zymograms the fastest reacting and darkest band was 9 band 3 appeared next and then band 5 Bands 2 and 3 on the other hand were the most reactive towards propionate and butyrate With these substrates band 1 was usually absent or very faintly positive and band 9 appeared as a double band.

Naphthol AS D acetate — Zymograms produced with this substrate showed band patterns different from those described above (Fig 35 and Colour fig 5) Only four bands exhibited activity towards this substrate they corresponded to bands 1 3 4 and 9 of naphthyl acetate preparations

Hydrolysis of Indoxyl Esters

If the originally prescribed histochemical procedure was used no reactive bands at all could be obtained with either *5 bromo* or *4 chloro 5 bromoindoxyl acetate* However when the concentration of the ferro ferricyanide redox buffer was lowered to 10 per cent of the recommended concentration the reaction became positive on the starch slab At pH 7 2 the reaction turned positive in four hours at 37 C Fewer bands were obtained than with naphthyl acetate and they were designated I1 to I4 (Fig 35 and Colour Fig 5) Bands I1 and I4 were weak and became positive only if the incubation time was prolonged

At pH 6 incubation overnight was needed before any bands could be seen Bands I1 and I4 were not reactive at this pH

Effect of Inhibitors

Thiocholine Esters

E600 eserine and *iso OMPA + 284C51* totally inhibited the hydrolysis of thiocholine esters. When acetylthiocholine was used with *iso OMPA* (10^{-6} or 10^{-5} M) to demonstrate the AChE activity alone the bands C1 C2 and C3 were obtained but the broad zone C4 was lacking (Fig 35) Butyrylthiocholine, either alone or with 10^{-5} M *284C51* produced the band C4 which therefore proved to be a ns ChE active zone (Fig 35)

Naphthol and Indoxyl Esters

In naphthyl acetate zymograms *284C51* abolished band 5 entirely and had a slight effect on bands 2 and 4 (Fig 35) An inhibitory effect on the zymograms of the other substrates could not be observed with certainty

The inhibitory effect of *iso OMPA* was seen in α naphthyl acetate and 4 chloro-5 bromoindoxyl acetate zymograms In the former it cleaned the background staining between bands 4 and 5 thus affecting band 6 selectively (Fig 35) In the latter only bands I1 I2 and I4 could be distinguished However when a more concentrated homogenate was used and incubation prolonged I3 appeared as two very weak lines I3a and I3b (Fig 35)

Eserine was always without effect on propionate butyrate and AS D acetate zymograms (Fig 35 and Colour Fig 5) In naphthyl acetate zymograms it in

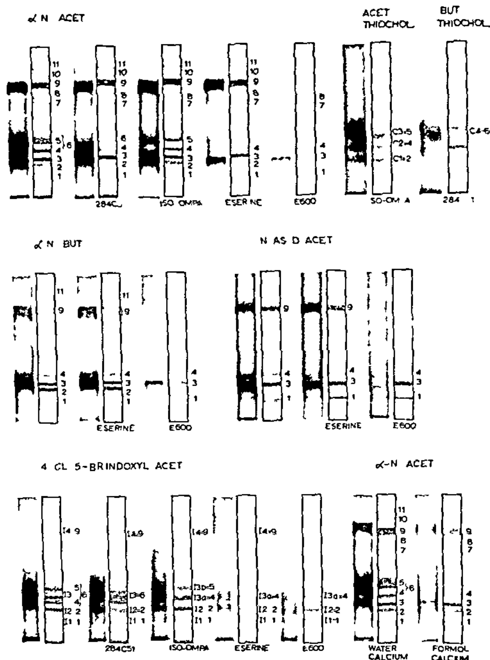


Fig 35 Esterase zymograms obtained with different substrates and inhibitors With each substrate all slabs have been obtained under strictly identical conditions of electrophoresis histochemical development and photographic reproduction Because the prints were developed so as to show also the weakest bands details of the strongly reacting bands have been lost

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Characterization of Substrate Inhibitor Properties of Different Bands

Comparisons between the bands in the zymograms of different substrate inhibitor combinations were made with neighbouring slices of the same starch slab or with zymograms obtained after simultaneous electrophoretic separation and staining In this way it was possible to characterize the enzyme activities of different bands and their substrate properties Of the substrates employed α naphthyl acetate was the only one showing all the bands demonstrable with any of the other substrates which were more selective as can be seen from Table 1 As was shown by experiments with inhibitors naphthol AS D acetate *demonstrated both E-s and E r ns E activity* but hardly any ChE activity whilst 4-chloro-5 bromoindoxyl acetate gave a positive reaction for ns ChE and E r ns E (prolonged incubation time) but showed very little ns E and AChE activity Acetylthiocholine gave positive reactions with ChEs alone and butyrylthiocholine demonstrated ns ChE exclusively α Naphthyl propionate and butyrate *did not demonstrate ChEs and not all of the E r ns E fractions* seemed to hydrolyse it

Correlation of Esterases in Sections and Zymograms

The proportions of ChEs and ns Es in the total esterase activity were different in zymograms and in fresh sections In fresh sections only a small part of the total activity demonstrable with α naphthyl acetate was eserine resistant (the activity in ganglion cells about 20 % of the total activity) and no E600 resistant activity could be demonstrated while in zymograms most of the activity developed with the same substrate was due to both E s and E r ns E activity

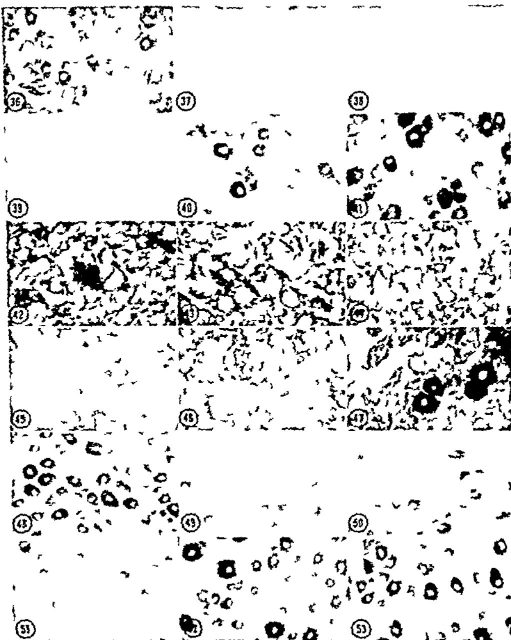
Strong reactions were also observed at the origin of the starch slab in the filter paper where the supernatant of the tissue homogenate was applied The partial inhibition of this reaction by iso OMPA and eserine and the total inhibition by E600 as well as the strong reactivity towards butyrylthiocholine indicate that the ns ChE and E-s ns E complex is predominant in the immobile fraction

In sections 284C51 had no clear inhibiting effect on the α naphthyl acetate reaction On the other hand in zymograms developed with the same substrate *the same inhibitor destroyed the activity in one band* completely (5) and weakened it in two others (2 and 4)

PREGANGLIONIC DENERVATION

Histochemical and Electrophoretic Observations

ChEs — Within the ganglion cells AChE and ns ChE activities were unchanged On the other hand AChE activity totally disappeared from the preganglionic axons and their terminals The remaining activity in the areas between the nerve cells was probably located in the postganglionic axons In this type of preparation it was clearly seen that the AChE activity *in some ganglion*



The effect of postganglionic denervation on different esterase activities × 120

Fig 36 AChE activity Fresh frozen section. Control ganglion. *Fig 37* AChE $1\frac{1}{2}$ days after axotomy *Fig 38* AChE, 3 days after axotomy *Fig 39* AChE 7 days after axotomy *Fig 40* AChE 50 days after axotomy *Fig 41* AChE 150 days after axotomy

Fig 42 NsChE activity Fresh frozen section Control ganglion *Fig 43* NsChE $1\frac{1}{2}$ days after axotomy *Fig 44* NsChE 3 days after axotomy *Fig 45* NsChE 7 days after axotomy *Fig 46* NsChE 50 days after axotomy *Fig 47* NsChE 150 days after axotomy

Fig 48 NsE activity α-Naphthyl acetate Fresh frozen section briefly postfixed in formol Macrodex. Control ganglion *Fig 49* NsE $1\frac{1}{2}$ days after axotomy *Fig 50* NsE 3 days after axotomy *Fig 51* NsE 7 days after axotomy *Fig 52* NsE 50 days after axotomy *Fig 53* NsE 150 days after axotomy

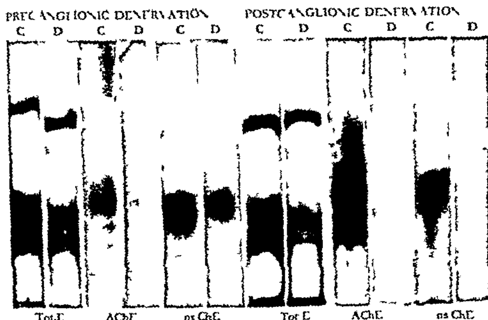


Fig 54 The effect of pre and postganglionic denervation on esterase zymograms Abbreviations C, control ganglion D denervated ganglion Tot.E total esterase (α-naphthyl acetate) AChE acetylcholinesterase ns ChE, non specific cholinesterase Each pair C and D has been obtained under strictly identical conditions from the same amount of fresh tissue

cells was concentrated at the cell membrane The first sign of the disappearance of AChE from the preganglionic fibres was seen 24 hours after operation and no activity was left after two days The ns ChE activity of the intercellular tissue was also somewhat decreased

In the zymograms preganglionic denervation to some extent weakened the AChE bands C2 and C3 and the ns ChE band C4 was also weaker than in the zymograms of the control side (Fig 54)

E s ns Es and *E r ns Es* — No differences in the activity of these esterases could be observed after preganglionic denervation The lyo-component of ns Es (= the activity of the intercellular tissue) was studied in formalin fixed preparations

The zymogram bands exhibiting E s and E r ns E activity were unchanged

POSTGANGLIONIC DENERVATION

Histochemical and Electrophoretic Observations

ChEs — Axotomy caused almost total disappearance of AChE from all the chromatolytic ganglion cells in two days (Figs 36 37 and 38) A decrease in the activity of this enzyme was already discernible 18 hours after axotomy There were always however some ganglion cells exhibiting AChE activity These cells presumably were not subjected to axotomy by the operation some

of the postganglionic fibres having escaped division During these two days a very strong AChE activity was observed in the distal ends of the axonal stumps (Fig 96) Some recovery was seen 50 days after operation when larger numbers of AChE positive cells were present in the ganglion (Fig 40) After 100—150 days many ganglion cells showed strong AChE activity (Fig 41)

Axotomy caused a loss of AChE activity also from intercellular structures i e from dendrites and preganglionic fibres (Figs 38 and 39)

Ns ChE normally positive in some neurones disappeared after axon division and the surrounding activity was also considerably weakened (Figs 42 43—45) No increased enzymatic activity such as was observed in AChE preparations could be seen in the axonal stumps The recovery of ns ChE activity in the ganglion (Figs 46 and 47) paralleled the recovery in the AChE activity

In the zymograms the AChE bands C1 and C2 were clearly weaker in axotomized ganglia than in control ganglia and the band C3 was usually totally absent (Fig 54) The activity of the ns ChE band C4 was very much decreased (Fig 54)

E s ns Es — This enzyme activity weakened in all chromatolytic cells The maximum decrease in activity was reached in 2—3 days (Figs 48 49 and 50) and this condition persisted for one — two months (Fig 51) after which the number of ns E-positive ganglion cells increased (Fig 52) By 100—150 days after the operation the picture was normal (Fig 53) In contrast to the ChE activities the E-s ns E activity of the chromatolytic nerve cells was never totally lost

E r ns Es — This activity was studied only 7—10 days after the operation in formalin fixed ganglia with naphthol AS D α naphthyl and 4-chloro-5 bromoindoxyl acetate as substrates No significant changes due to the operation could be observed

In the α naphthyl acetate zymogram, the bands exhibiting E s or E-r ns E activity were not affected, except for band 3 which was somewhat weaker than that in the control zymogram (Fig 54)

Quantitative Determinations

The determinations of esterase activities were carried out between the 7th and 13th postoperative days In the present study the right superior cervical ganglion was subjected to axotomy and the left one served as a control The results were calculated as total esterase ChE, E-s ns E and E-r ns E activities From 30 normal ganglia the relative proportions of different esterases were the following expressed as per cent of total esterase activity ChE 61 2 % (standard deviation 7 3 %) E s ns E 31 2% (SD 7 7%) E-r ns E 7 6% (SD 3 3%)

The differences between the enzyme activities of the intact right and left ganglia (control group) as well as between the axotomized right ganglia and the normal left ganglia (axotomy group) are expressed in the form of regression equations $y = kx$ where y stands for the activity of the right side and x for that of the left side The significance of the difference between the regressions was tested using Fischer s F test by comparing the residual variances in the control and axotomy groups

Total Esterase (µM β naphthol/mg protein/hour)

Control $y = 1\ 04\ x$
Axotomy $y = 0\ 50\ x$
Total $y = 0\ 74\ x$

Analysis of variance

Group	Sums of squares			f	s_0^2	F	$P<$
	Q	Q_R	Q_a				
Control	239 22	232.77	6 45	8	0 806	65 58	0 001
Axotomy	74 18	71 60	3 58	11	0 326		
Total	313 40	268 75	44 75	20	2 235		

CbE (µM β naphthol/mg protein/hour)

Control $y = 1\ 04\ x$
Axotomy $y = 0\ 39\ x$
Total $y = 0\ 62\ x$

Analysis of variance

Group	Sums of squares			f	s_0^2	F	$P<$
	Q	Q_R	Q_b				
Control	80 84	79 01	1 83	8	0 229	87 45	0 001
Axotomy	22 88	20 43	2 45	11	0 223		
Total	103 72	79 74	23 98	20	1 199		

E s ns E (μM β naphthol/mg protein/hour)

Control $y = 1\ 06\ x$
Axotomy $y = 0\ 67\ x$
Total $y = 0\ 90\ x$

Analysis of variance

Group	Sums of squares			f	s_0^2	F	$P<$
	Q	Q_R	Q_0				
Control	34 67	31 54	3 14	8	0 397	9 69	0 01
Axotomy	9 47	9 04	0 43	11	0 039		
Total	44 14	38 75	5 39	20	0 270		

E r ns E (μM β naphthol/mg protein/hour)

Control $y = 1\ 39\ x$
Axotomy $y = 0\ 86\ x$
Total $y = 1\ 13\ x$

Analysis of variance

Group	Sums of squares			f	s_0^2	F	$P<$
	Q	Q_R	Q_0				
Control	2 49	2.22	0 28	8	0 035	6 19	0 05
Axotomy	1 04	0 82	0 22	11	0 020		
Total	3 53	2 88	0 65	20	0 033		

Axotomy caused the following percentage losses as judged from the left right difference of the original enzyme activity total Es 50 % ChEs 61 % E-s ns E 33 % E r ns E 14 %

DISCUSSION

Bound and Soluble Esterases

In earlier quantitative studies on the enzyme activity in tissue homogenates it was observed that part of the activity could easily be extracted while the remainder appeared to be bound to the cellular debris. This observation resulted in the introduction of the terms lyo- and desmo-enzymes, the former indicating the soluble component and the latter the bound fraction (Willstatter and Rohdewald 1932, 1933, Bamann and Laeverenz 1934).

In histochemical localization of enzyme activities the soluble component may diffuse into the incubation solution and thus fail to indicate the site of the original activity. According to Nachlas and his co-workers (Nachlas et al. 1956, Hannibal and Nachlas 1959) the desmo-component of the rat kidney esterases in fresh sections represents about one third of the total esterase activity.

Since fresh sections, therefore, give an incomplete idea of the total esterase activity, the only possibility is to immobilize the lyo-esterases and thus make them accessible to histochemical demonstration. Formalin fixation in the present study brought about a positive reaction in areas which had been entirely negative in fresh sections. In formalin fixed ganglia E-r-ns-E, which was absent from fresh sections, showed a strong activity in the small oval cells and a weaker activity in the ganglion cells. Similarly, the E-s-ns-E reaction in the interstitial tissue of postfixed sections and formalin fixed ganglia seems to be at least partly dependent on immobilization of the originally soluble enzyme by formalin. On the other hand, the E-s-ns-E activity responsible for the reaction in the cytoplasm of the ganglion cells of unfixed and briefly postfixed sections was weakened by long formalin fixation so that the activities in the cytoplasm of the ganglion cells and in the interstitial tissue were equal.

Readily soluble esterases have been shown to produce zymogram patterns essentially similar to those obtained with homogenates but different from those obtained with solubilized desmo-esterases firmly attached to the tissue (Eranko et al. 1964). It was observed that most of the total E-s-ns-E activity in the desmo-zymogram was limited to the so-called Ch region (= region 6 in the present study) and most of the similar enzyme activity in the lyo-zymogram was found in the so called ns zone (= region 9—11 in the present study).

Although in the present study formalin had an inhibiting effect on the ChE bands 5 and 6 in which no E-s-ns-E activity was found, such activity is demonstrable in region 6 of desmo-zymograms (Eranko et al. 1964). Therefore it is reasonable to suppose that this zone contains the formalin sensitive E-s-ns-E activity of the ganglion cells demostrable in fresh sections, while the soluble interstitial activity demonstrable in formalin fixed sections only may correspond to similar activity in band 9.

With thiocholine as substrate and free floating sections transferred directly to the incubation solution after cutting from fresh and from formalin fixed ganglia, it was observed that formalin caused only a slight decrease of ChE activity, AChE being more sensitive. Similar observations had previously been reported by Fukuda and Koelle (1959) and by Lewis (1961). In the zymograms developed with α-naphthyl acetate the bands which proved to contain both ChEs hydrolysing α-naphthyl acetate were completely inactivated by formalin

E s ns E (μM β naphthol/mg protein/hour)

Control y = 1 06 x
Axotomy y = 0 67 x
Total y = 0 90 x

Analysis of variance

Group	Sums of squares			f	s_0^2	F	$P<$
	Q	Q_R	Q_0				
Control	34 67	31 54	3 14	8	0 397	9 69	0 01
Axotomy	9 47	9 04	0 43	11	0 039		
Total	44 14	38 75	5 39	20	0 270		

E r ns E (μM β naphthol/mg protein/hour)

Control y = 1 39 x
Axotomy y = 0 86 x
Total y = 1 13 x

Analysis of variance

Group	Sums of squares			f	s_0^2	F	$P<$
	Q	Q_R	Q_0				
Control	2 49	2.22	0 28	8	0 035	6 19	0 05
Axotomy	1 04	0 82	0 22	11	0 020		
Total	3 53	2 88	0 65	20	0 033		

Axotomy caused the following percentage losses as judged from the left right difference of the original enzyme activity total Es 50 % ChEs 61 % E-s ns E 33 % E r ns E 14 %

as a substrate an intense 284C51 sensitive AChE activity was observed in all nerve cells and preganglionic fibres Intense ns E activity may in the cells mask AChE activity towards α naphthyl acetate Since iso-OMPA in interstitial tissue and in preganglionic nerve fibres of fresh sections totally destroyed all the activity towards α naphthyl esters it is quite clear that AChE in these fibres which are known to possess considerable AChE activity cannot hydrolyse naphthyl esters

In the zymograms developed with α naphthyl acetate three bands corresponded to the three bands seen in the AChE zymogram but only the fastest moving of these three was totally inhibited by eserine and 284C51 At the levels of the other two bands one must therefore suppose multilocus type esterases capable of hydrolysing acetylthiocholine as well as other acetate esters or two different enzymes moving in an electric field at the same rate No E600 resistant esterases were found capable of hydrolysing thiocholine substrates in the manner described by Vanha Perttula and Hopsu (1964) in the rat pituitary gland

Distribution of Esterases in the Ganglion

It is difficult *to determine accurately the actual site of esterase activity in the* structures between the ganglion cells because this interstitial structure consists of so many tissue components pre and postganglionic nerve fibres capsular and Schwann cells and dendrites In the vicinity of the ganglion cells were strongly esterase positive »tentacles» most of which have been shown to be dendrites by electron microscopy (Elfvin 1963a b)

The division of the preganglionic nerve trunk had only a little effect on the total E and ns ChE activity in the intercellular fibres whereas axotomy caused a clear decrease in their esterase activity which lends support to the view that these structures originate from the ganglion cells However the preganglionic fibres also showed clearly diminished AChE activity

On the other hand Sjoqvist (1962) has suggested that the surrounding ns ChE positive interstitium is mainly a network of capsular and Schwann cells and that the decreased activity in these cells after axotomy may result from the action of some unspecific factors such as hypoxia While undoubtedly many of the ns ChE positive structures are capsular and Schwann cells the proposed explanation of the dendritic location of this enzyme awaits further evidence

Significance of Cholinesterases

The significance of strong ns ChE activity in the glial cells is not clear It was suggested by Koelle (1962) that it may play some part in hydrolysing the AChE released in synapses Outside the nervous system ns ChE should hydrolyse toxic acyl choline compounds (mainly butyrylcholine) which may be formed by accident in fatty acid oxidation from acyl CoA and choline (Clitherow et al 1963)

The role of AChE in adrenergic neurones is still unknown Burn and Rand (1960 1962) have proposed that a cholinergic mechanism intervenes in the release of noradrenaline by postganglionic adrenergic fibres They concluded

that adrenergic nerve impulses first liberate ACh at the terminals and that this in turn releases noradrenaline. The low concentration of AChE observed in various sympathetic ganglion cells may be explained by this theory As far as the postganglionic fibres can be traced their level of AChE activity is proportional to that of their neurones of origin (Koelle 1955 Giacobini 1957) This presupposes however that the different levels of AChE in the cell body always reflect the corresponding extent of participation of ACh in transmission Although the high concentrations of ACh, ChA and AChE correspond well to each other in the cholinergic neurones there is still no direct evidence of the presence of either ACh or ChA in adrenergic neurones (Hebb 1963)

Nachmansohn (1959) has also presented a hypothesis of the universal role of ACh in transmission based on his biochemical findings that AChE occurs in all nerves in concentrations that are adequate to maintain conduction.

Other hypotheses concerning the same problem have been presented For instance there might be another unknown substrate for AChE in adrenergic nerves which might or might not be associated with the process of conduction (Feldberg 1957) Alternatively cholinergic neurones may be phylogenetically older and in the course of evolution to non-cholinergic neurones the fibres have lost their ChA but not their AChE, which remains as a kind of biochemical vestige (Koelle 1955 Feldberg 1957)

Fredricsson and Sjöqvist (1962) concluded that the strongly as well as the moderately AChE-positive nerve cells in the sympathetic ganglia of the cat were cholinergic, while the cells with very weak activity were adrenergic. After axotomy the authors observed the disappearance of the first mentioned cell types whereas the activity in the adrenergic cells was unaffected The preganglionic fibres also showed decreased AChE activity but BuChE activity was not affected by the operation.

In the present study postganglionic axotomy resulted in almost total disappearance of AChE in the chromatolytic ganglion cells Ns ChE apparently suffered the same fate because positive cells were less frequent than in the control ganglia Because all the neurones in the superior cervical ganglion of the rat are normally clearly AChE-positive there was no doubt about the disappearance of the activity in the adrenergic neurones too

Significance of Non-specific Esterases

The function of the ns Es in the sympathetic ganglion or anywhere else for that matter is completely unknown. Ali-esterases (= ns Es) of serum and many tissues of higher animals could be inhibited in vivo almost completely without affecting the well being and growth of the animals (Mendel et al. 1953) Nor could any changes be observed in the normal lipid metabolism of the rat liver (Myers and Mendel 1953) On the other hand the growth of the lower forms of life (seeds tubercle bacilli malignant cells) could be arrested by inhibition of their ns E Insecticidal action of organophosphates has been explained to be at least partly due to the inhibition of ns Es (van Asperen 1958 Smith and Wagenknecht 1959) Therefore it has been suggested that in the course of evolution the ns Es may have lost their significance in the metabolism of higher animals (Myers and Mendel 1953)

The finding that ns Es are capable of hydrolysing amides and amino acid esters suggests that they may act as proteinases (Myers et al 1957 Hopsu and Glenner 1964a b) This is supported by the observation that some of the multiple bands of proteolytic and esterolytic enzymes appear to share an identical location on the zymogram (Hunter et al 1964) Evidently some of the esterases classified in this group are capable of hydrolysing certain kinds of peptide bonds but there are also esterases which evidently *do not* (Vanha Perttula *and Hopsu* 1964) The partial decrease of ns E activity in chromatolytic ganglion cells might thus indicate a delayed protein catabolism It has been found indeed that in the nerve cells during the axon reaction efficient production of proteins and lipids occurs (Brattgård et al 1957)

that adrenergic nerve impulses first liberate ACh at the terminals and that this in turn releases noradrenaline The low concentration of AChE observed in various sympathetic ganglion cells may be explained by this theory As far as the postganglionic fibres can be traced their level of AChE activity is propor tional to that of their neurones of origin (Koelle 1955 Giacobini 1957) This presupposes however that the different levels of AChE in the cell body always reflect the corresponding extent of participation of ACh in transmission Al though the high concentrations of ACh ChA and AChE correspond well to each other in the cholinergic neurones there is still no direct evidence of the presence of either ACh or ChA in adrenergic neurones (Hebb 1963)

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Histochemical demonstration of *monoamine oxidase* (MAO) activity is based either upon the reduction of tetrazolium salts when the amines are oxidized (Glenner et al 1957) or on the demonstration of aldehydes formed by amine oxidation (Koelle and Valk 1954 Eder 1957) The tetrazolium procedure of Glenner gives easily reproducible results Indolyl 3-acetaldehyde formed as the result of oxidative deamination of tryptamine has been shown to reduce sensitive tetrazolium salts directly and in this case the pigment formed should indicate the exact localization of the enzyme even intracellularly (Glenner et al. 1960)

DISTRIBUTION OF OXIDATIVE ENZYMES IN NERVOUS TISSUE

Dehydrogenases

Histochemical studies on dehydrogenase activity in nervous tissue carried out with the older tetrazolium salts are of little but historical interest (Leduc and Wislocki 1952, Padykula 1952, Rutenburg et al. 1953 Mustakallio 1954)

When more suitable tetrazolium salts became available succinate dehydrogenase activity was demonstrated in almost all nerve cells and their processes in both the human and animal central nervous system glial cells have also been reported to show such activity (Nachlas et al. 1957 Shimizu and Morikawa 1957 Shimizu et al. 1957 Potanos et al 1959) Friede (1959a b c, 1960 1961) published an extensive study in which succinate dehydrogenase activity was mapped in the entire brain of the guinea-pig In subsequent papers (1962 1963) he also studied other oxidative enzymes in human and monkey brain (lactate glucose 6-phosphate 6-phosphogluconate DPN and TPN diaphorase and cytochrome oxidase) According to him, »the patterns of different enzymes were almost identical among most of the nuclei but marked differences could be noticed within any given nucleus »

A wide variation in the activity and intracellular distribution of various oxidative enzymes has been found in individual spinal ganglion cells (Samorajski 1960 Thomas and Pearse 1961 Tewari and Bourne 1962a b) In the trigeminal ganglion cells a similar variation in enzymatic activity has been observed (Tewari and Bourne 1963b) Tewari and Bourne suggest that these variations reflect changes in the nerve cells which undergo continuous metabolic cycles related to the synthesis of enzymes and other proteins (1962a 1963b)

Different patterns of enzymatic activity in the central nervous system have been obtained with MTT and NBT as hydrogen acceptors With MTT a low glial activity was observed and the conflicting results obtained with NBT were attributed to the »substantivity» of this salt on the glial cell proteins (Thomas and Pearse 1961) Concerning the neurones it was concluded that MTT primarily localizes the intramitochondrial diaphorase activity while NBT demonstrates both intra and extramitochondrial oxidative pathways (Samorajski 1960)

A series of microchemical investigations on enzymes in the central nervous system has been published by Lowry and his associates (Robins and Smith 1953 Strominger and Lowry 1955 Robins et al. 1956 Buell et al. 1960 Robins 1960) The enzymes of the glycolytic and citric acid cycles exhibited their highest activity in the molecular cortex and their lowest activity in the white matter In contrast to this glucose-6-phosphate dehydrogenase was lowest in the granular cortex The molecular layer of the cerebellar cortex is composed largely of cell processes whereas in the granular layer there is a preponderance of cell bodies. Therefore the results suggest that the activities of these enzymes are generally more characteristic of cell processes than of cell bodies and that relatively little glucose is metabolized via the pentose cycle

Pope and his collaborators (Pope et al 1956 Pope 1958) stated that the respiratory enzymes were localized mainly to the neurones in the cerebral cortex. However it has been reported that the succinate dehydrogenase activity in the grey matter is lower than in the white matter when the tetrazolium reductase method is used on brain homogenate (Kawakita 1956)

Hyden and his co-workers (Hydén et al. 1958 Hyden and Pigon 1960) working with isolated spinal ganglion cells or Deiters nerve cells and their capsules containing satellite cells found higher respiratory enzyme activity (succinate dehydrogenase and cytochrome oxidase) per unit volume in the glial cells than in the nerve cells Manometric microdeterminations of oxygen consumption also showed that succinate and pyruvate are essentially oxidized by the glia whereas the nerve cell bodies mainly utilize glutamate (Hamberger 1961)

Lowry and his colleagues (Lowry et al. 1956 Lowry 1957a b) when analysing the enzyme activity of individual spinal ganglion cells and their glial capsules on a dry weight basis found that in comparison with the nerve cell bodies the capsules are very low in hexokinase malic dehydrogenase transaminase and glutamic dehydrogenase about equal in phosphoglucoisomerase lactic dehydrogenase and isocitric dehydrogenase and relatively rich in glucose-6-phosphate and 6-phosphogluconate dehydrogenase However if these results are recalculated on a volume basis the relative activities in the smaller glial cells are much higher than those of the larger nerve cells (Hyden and Pigon 1960 Hyden 1960)

Enzyme activities in the axon sheath of the squid giant fibre are generally lower than those in the axoplasm (Roberts et al 1958) However oxygen consumption and the activities of the enzymes of the citric acid cycle have been shown to be higher in the sheath cells than in the axon (Coelho et al. 1960 Roberts et al. 1958)

On the other hand, macrochemical studies have shown that oxidative metabolism in the neuroglia is lower than in the neurones (Abood et al. 1952 Elliott and Heller 1957) Histochemically DPN diaphorase activity was observed to be more intense in the neurones while that of TPN diaphorase predominated in the satellite cells and when the dehydrogenase systems were arranged in order of decreasing activity in the neurones the activity was increased in the satellite cells (Romanuel and Cohen 1960)

Cytochrome oxidase

The enzyme indophenol oxidase was first demonstrated in nerve cells by Pighini (1912) The nervous system was systematically studied by this technique and the identity of corpuscles with mitochondria was established (Marinesco 1924 Bielschowsky and Rose 1927) A positive reaction was reported in autonomic ganglion cells and capsular cells among other locations (Katsunuma 1915)

In the mammalian brain, a positive histochemical reaction was found in all layers except those consisting of white matter however the different regions of the grey matter showed differences in activity (Shimizu et al. 1957 Nachlas et al. 1958c Burstone 1960)

An especially intense reaction has been reported in the trigeminal ganglion (Burstone 1961) In the spinal ganglion some cells have been reported to be completely negative others being positive in the latter the reaction was seen around the nucleus or was associated with the cell membranes (Tewari and Bourne 1962a) The amount and distribution of Nissl material in the nerve cells has been claimed to parallel the intensity of the indophenol oxidase reaction (Roizin 1955)

Quantitative histochemical investigations concerning the distribution of cytochrome oxidase in different layers of the brain indicate that this enzyme is primarily localized in the

mitochondria of the nerve cell bodies and dendrites rather than in the axons or neuroglial elements (Pope et al. 1956 1957) The histochemical pattern of indophenol oxidase activity in the brain cortex is in agreement with this opinion (Meath and Pope 1950)

Monoamine oxidase

Nerve cell bodies and fibres of different adrenergic ganglia of the cat were found to exhibit similar levels of MAO activity (Koelle and Valk 1954) However in the same study it was observed that the ganglion cells of the adrenergic neurones of the rabbit exhibited considerably stronger MAO reaction than did the sensory or cholinergic neurones. In guinea pig tissues Auerbach s and Meissner s plexuses were intensely stained (Eder 1957)

Sympathetic ganglia of the guinea-pig were reported by Glenner et al. (1957) to be intensely stained when the tryptamine tetrazolium procedure was used. Myelinated nerve fibres were found to be negative The distribution of MAO activity demonstrable with this method was said to give results identical with those obtained with the Koelle Valk technique

MTT in the presence of metal ions has also been used to demonstrate MAO activity and intramitochondrial localization of the enzyme has been suggested (Wohlrab 1961)

The Koelle Valk method is not readily applicable to the study of the central nervous system because of an over-all staining even in the absence of the substrate due to a direct reaction between lipids and naphthoic acid hydrazide even after pretreatment with non-chromogenic hydrazines Therefore other histochemical techniques have been employed for MAO localization in the brain. The nerve cells in the hypothalamic nuclei and the basal ganglia as well as the Purkinje cells in the cerebellum of the mouse and rat have been reported to be positive when the formation of brown pigment from 5-HT (Arioka and Tanimukai 1957) or the tetrazolium method (Tewari and Bourne 1963 a) has been used to visualize MAO activity The distribution of MAO in the brain of some adult rodents was described by Shimizu et al. (1959) An inverse relationship between the activity levels of MAO and succinate dehydrogenase or cytochrome oxidase was observed The authors concluded that MAO may be involved in the metabolism of the visceral regions of the brain rather than in the function of the adrenergic neurones

The results of biochemical estimations of MAO activity in nervous tissues are largely in agreement with those obtained with the aid of histochemical staining reactions Especially intense MAO activity in the thalamic and hypothalamic regions of mammalian brains has been revealed by biochemical determinations (Pugh and Quastel 1937 Birkhauser 1940 Bogdanski and Udenfriend 1956 Bogdanski et al 1957 Weiner 1960)

Effect of Denervation on Oxidative Enzyme Activities

Studies concerning the oxidative enzymes during the axon reaction are surprisingly few and the results are conflicting All the studies as yet reported are briefly summarized in the following survey

A decreased succinate dehydrogenase activity was found biochemically in the ventral grey column following section of the sciatic nerve (Howe and Flexner 1947) A similar observation was made by Friede (1959d) using histochemical techniques for succinate dehydrogenase and DPN and TPN-diaphorases The depletion of enzymes from the nerve cells was most pronounced for succinate dehydrogenase and TPN-diaphorase. A decrease in succinate dehydrogenase activity was also observed in the nerve cell bodies of the facial nucleus during chromatolysis (Coimbra 1961)

In contrast to the above reports Kreutzberg (1963) who studied the nucleus facialis of the guinea pig after transsection of the facial nerve observed a significant increase in the TPN diaphorase and glucose 6-phosphate dehydrogenase activities in the ganglion cells 5—50 days after operation Changes in the activity of DPN-diaphorase and DPN linked dehydrogenases were not so marked A similar increase was observed by Klein (1960) in the succinate dehydrogenase activity of spinal ganglion cells after transsection of the sciatic nerve An increased succinate dehydrogenase and DPN and TPN-diaphorase activity was found in tissue cultures of dorsal root ganglia during the second week after the beginning of culture which naturally included only nerve cells with divided axons (Yonezava et al. 1963) The transneuronal atrophy after enucleation of the eye on the other hand has been found to cause a marked decrease in succinate lactate and 6 phosphogluconate dehydrogenase as well as DPN and TPN-diaphorase activities in the geniculate nuclei (Kupfer 1963)

Howe and Mellors (1945) made a manometric estimation of the cytochrome oxidase activity of the spinal cord after transsection of the peripheral nerves An almost 20 % reduction in activity was observed but no correlation with the degree of chromatolysis could be established Histochemically demonstrable cytochrome oxidase activity has also been reported to decrease in chromatolytic nerve cells (Roizin 1951)

METHODS

Preparation of Tissues

The preparative procedures used have been described in detail under *General Material and Methods* Fresh cryostat sections and sections cut from minute blocks fixed in graded concentrations of chilled formalin for a short time (Walker and Seligman 1963) were used for histochemical studies The section thickness was 6—10μ

Histochemical Methods

NADH and NADPH Tetrazolium Reductases — The medium described by Scarpelli Hess and Pearse (1958) was used NBT TNBT and MTT were used as final electron acceptors The stock solution was kept frozen at —30 C for several weeks The incubation time was 20 minutes

NAD linked Tetrazolium Reductases — The substrates used were α glycerophosphate lactate β-hydroxybutyrate isocitrate malate and glutamate The stock solution was prepared according to Hess et al (1958) Each incubation medium made up just prior to use consisted of 0 9 ml of stock solution 0 1 ml of 1 M substrate and 6 mg NAD Incubation times were 20 minutes for lactate tetrazolium reductase and 30 minutes for the other enzymes

NADP linked Tetrazolium Reductases — Glucose-6-phosphate and isocitrate were used as substrates for demonstrating these enzymes according to Hess et al (1958) The incubation time was 30 minutes

Succinate Tetrazolium Reductase — This enzyme was demonstrated according to Nachlas et al (1957) The incubation time was 45 minutes

When phenazine methosulphate (PMS) was added to the incubation medium its final concentration was 20—50 μg ml The incubation times were the same or 15 minutes shorter than without PMS

For the demonstration of *[illegible]-menadione* and *[illegible]* menadione (2-methyl-1,4-naphthoquinone) dissolved in acetone was added so that the final concentration in the reaction mixture was 0.1 mg/ml. The incubation time was 30 minutes.

Incubation was stopped by rinsing the section and fixing for 30–60 minutes in formol Macrodex. The sections were washed in distilled water and mounted in glycerin jelly. Control sections incubated in the absence of substrate were negative.

Cytochrome Oxidase (CyO) — This was demonstrated according to Burstone (1959, 1960, 1961) using 8-amino-1,2,3,4-tetrahydroquinoline (Eastman Kodak, Rochester, New York) as coupler. Two mg of cytochrome c was added to 5 ml of incubation solution and the incubation was carried out at room temperature for 40–60 minutes. After incubation postchelation was performed in 10% cobaltous acetate formalin solution. To study the specificity of the staining reaction, potassium cyanide was added to the incubation solution at a final concentration of 10^{-3} M. The staining reaction was completely inhibited by this concentration of cyanide.

Monoamine Oxidase (MAO) — This enzyme was demonstrated both with the tryptamine tetrazolium method of Glenner et al. (1957) and the hydrazone precipitation technique of Koelle and Valk (1954).

In the former method NBT or TNBT served as final electron acceptor and sodium sulphate was omitted from the incubation solution. Incubation was carried out at 37° C for 50–60 minutes.

The original method of Koelle and Valk (1954) was modified in the following way: the preincubation solution was prepared from 10 ml of 0.1 M phosphate buffer, pH 7.6, 10 ml of 40% Na_2SO_4 and 2 ml of 0.1 M hydrazine hydrochloride. The sections were preincubated in this solution for one hour at room temperature and thereafter rinsed several times in a solution consisting of equal volumes of 40% Na_2SO_4 and 0.1 M phosphate buffer. The incubation medium consisted of 10 ml of 0.1 M phosphate buffer and 10 ml of 40% Na_2SO_4. The medium was saturated with naphthoic acid hydrazide (NAH) at 80–90° C, cooled and filtered, and 20 mg of tryptamine hydrochloride was added. Incubation was carried out at 37° C for 2 hours with oxygen bubbling through the solution. After rinsing in water the slides were developed in Fast Blue B solution (300 mg Fast Blue B in 15 ml of 0.1 M phosphate buffer, pH 7.6) for three minutes.

Isonicotinyl-2-isopropyl hydrazine phosphate (Marsilid) was used as a selective inhibitor for MAO and its final concentration in the preincubation and incubation solutions was 10^{-4} M. Controls were tested without substrate.

Quantitative Determinations

Lactate Dehydrogenase (LD) — The assay technique was based principally on the method of Wroblewski and La Due (1955) and modified as described by Bergmeyer et al. (1962). The reaction mixture consisted of 150 µl of phosphate buffer (0.1 M, pH 7.4), 10 µl of NADH (3.2×10^{-3} M 1% $NaHCO_3$), 10 µl of homogenate and 30 µl of sodium pyruvate (2.4×10^{-2} M). After addition of pyruvate the decrease of optical density was followed at 30 second intervals for 5 minutes.

Malate Dehydrogenase (MD) — The determination was based on the method used by Ochoa (1955). Sodium oxalacetate was freshly prepared by neutralizing 4.1×10^{-3} M oxalacetic acid with sodium hydroxide. The concentrations of homogenate and incubation medium were the same as in the LD determination.

Glucose-6-Phosphate Dehydrogenase (G-6-PD) — The method employed was essentially that

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of Kornberg and Horecker (1955). The final volume of reaction mixture was 200 µl and consisted of 150 µl of TRA buffer (triethanolamine buffer 5×10^{-2} M, pH 7.6 and EDTA 5×10^{-3} M), 10 µl NADP (10^{-2} M), 30 µl of homogenate supernatant and 10 µl of glucose-6-phosphate (2×10^{-2} M). After preincubation for 5 minutes, G-6-P was added. The readings were taken at 30 second intervals at 340 mµ in a Beckman DU spectrophotometer against a blank from which the substrate was omitted. The linear change in optical density was followed for 5 minutes.

NADH Cytochrome c Reductase (RED). — The determination was made essentially according to Mahler (1955) and to Brady et al. (1952). The reaction mixture consisted of 150 µl of phosphate buffer (0.067 M, pH 7.4), 20 µl of ferricytochrome c (8.4 mg/ml distilled water), 10 µl of NADH (1.4×10^{-3} M/1 % $NaHCO_3$), 10 µl of potassium cyanide 0.02 M) and 10 µl of homogenate. The change in optical density was determined at 550 mµ at 15 second intervals for three minutes.

The protein content of the homogenates was determined by the method of Lowry et al. (1951). Bovine serum of known protein content was used as a standard.

Enzyme activities were expressed as µM of NAD, NADP and cytochrome c converted per minute and were referred to the protein content (mg) of the ganglion.

RESULTS

NORMAL GANGLION

Histochemical Observations

NADH Tetrazolium Reductase (DPN diaphorase). — All the neurones showed cytoplasmic activity to a varying degree. There was no correlation between the intensity of the enzyme reaction and the size of the nerve cells. With NBT the diformazan deposit was homogeneous within the ganglion cells. However, in thin sections and after a short incubation the reaction was observed to be also localized in small granules, which stood out clearly against the diffusely stained background. The nucleus and the nucleolus were always negative. The reaction was weak and mostly finely granular in the nerve fibres. In some thick myelinated fibres the myelin sheath was stained. In the capsular cells only some intensely blue granules were observed.

If TNBT was used instead of NBT, the distribution of the reaction was similar but the cytoplasmic granules seemed to be more selectively demonstrated (Fig. 55). Because of the brownish grey colour of the reduced TNBT it was difficult to estimate the relative intensity of the reaction. Crystallization phenomena or artifacts caused by lipid droplets, sometimes observed with NBT, never occurred in TNBT stained sections.

Fixation with formalin in Hanks balanced salt solution (BSS) resulted in only a slight decrease in activity and the granular distribution of the enzyme activity was better demonstrated than in fresh sections (Fig. 56). These granules probably represented mitochondrial activity of NADH tetrazolium reductase.

With the MTT cobalt method, the sites of activity appeared in the form of small formazan granules throughout the cytoplasm of the nerve cells (Fig. 57). The nerve fibres and capsular cells showed hardly any reaction. Because of the

coarse precipitate it was not possible to obtain a precise idea of the site of activity in the cytoplasmic elements

NADPH Tetrazolium Reductase (TPN diaphorase) — The reaction was positive in all ganglion cells but the staining was lighter than with NADH as substrate The reaction was localized in granules as well as diffusely and the intensity of staining varied somewhat in different cells (Fig 58) The nuclei were unstained The nerve fibres and capsular cells were stained in the same way as in NADH preparations

Formalin fixation reduced the diffuse cytoplasmic staining so that the granules were even more clearly seen

Succinate Tetrazolium Reductase — The reaction was weak but clearly positive in all ganglion cells Small positive granules were seen throughout the cytoplasm and no diffuse staining was seen unless freezing of the tissue was carried out slowly or the block was allowed to thaw before cutting In a few cells the granules displayed a tendency to concentrate around the unstained nucleus The capsular cells also showed a reaction localized in the cytoplasmic granules which reacted intensely The nerve fibres reacted weakly except for the few myelinated ones TNBT produced a similar picture (Fig 63) but with the MTT-cobalt technique the reaction was almost negative.

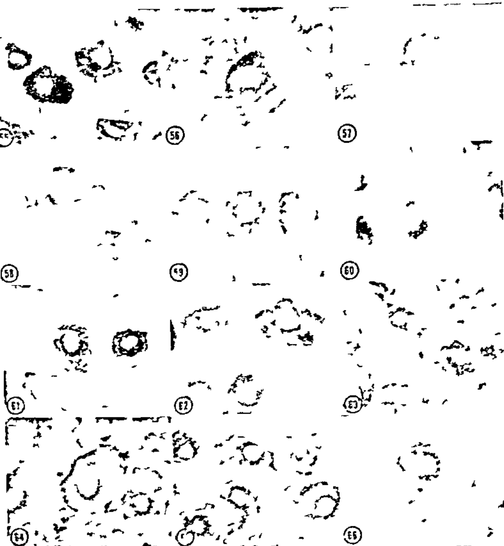
Addition of PMS to the incubating medium enhanced the reaction without affecting its distribution The effect of menadione was similar but more pronounced Even a short fixation in formalin abolished the enzyme activity completely

NAD Linked Tetrazolium Reductases — All components of the superior cervical ganglion reacted very weakly towards *α glycerophosphate* PMS had no significant effect on the reaction On the contrary when menadione was used as intermediate electron acceptor and NAD was omitted an intense granular reaction was seen in the cytoplasm of the nerve cells while the capsular cells showed a much weaker reaction In most ganglion cells the positive granules were concentrated in the perinuclear region thus enzyme thus differing from succinate tetrazolium reductase in its distribution (Fig 79)

The reductase activities towards *sodium l lactate* (Fig 67) and *l malate* (Fig 77) were quite similar to that of NADH tetrazolium reductase When thin sections were used a granular reaction was seen besides diffuse cytoplasmic staining in the ganglion cells Cold formalin fixation somewhat weakened the reaction but the distribution remained the same (Fig 59)

With both NBT and TNBT a moderately positive *β hydroxybutyrate tetrazolium reductase* reaction was seen in small granules of the ganglion cell often concentrated around the nucleus and weak diffuse cytoplasmic staining (Fig 61) The glial cells showed especially intensely stained cytoplasmic granules After formalin treatment the reaction was weaker but otherwise similar The activity was very weak with the MTT cobalt method

Isocitrate tetrazolium reductase activity varied greatly from one individual nerve cell to another (Fig 60) Some of the ganglion cell were negative and in the strongly positive cells the intracellular distribution of the reaction was sometimes uneven and selectively concentrated in a limited area of the cytoplasm The reaction was finely granular or homogeneous and positive also in the



Oxidative enzyme activities. x 390

Fig 55 NADH tetrazolium reductase, TNBT fresh frozen section.
Fig 58 NADPH tetrazolium reductase, NBT fresh frozen section.
Fig 61 β-Hydroxybutyrate tetrazolium reductase NBT fresh frozen section.
Fig 64 Cytochrome oxidase fresh frozen section.

Fig 56 NADH tetrazolium reductase NBT fixed for 13 min. in BSS
Fig 59 Lactate tetrazolium reductase, NBT fixed for 13 min. in BSS
Fig 62 Glucose-6-phosphate tetrazolium reductase NBT fresh frozen section.
Fig 65 Monoamine oxidase (Glenner et al.) fresh frozen section.

Fig 57 NADH tetrazolium reductase MTT fresh frozen section.
Fig 60 Isocitrate tetrazolium reductase (NAD) NBT fixed for 8 min in BSS
Fig 63 Succinate tetrazolium reductase TNBT fresh frozen section
Fig 66 Monoamine oxidase (Koelle Valk) fresh frozen section.

satellite cells and nerve fibres After very short formalin fixation the reaction was still positive (Fig 60) but longer exposure abolished it totally

Glutamate tetrazolium reductase activity could not be demonstrated in the superior cervical ganglion of the rat with any of the tetrazolium salts

NADP Linked Tetrazolium Reductases — *Glucose 6 phosphate tetrazolium reductase* (Figs 62 and 73) showed a diffuse cytoplasmic staining of the ganglion cells Nerve fibres were weakly positive and the activity seen in the surrounding capsular cells was located in cytoplasmic granules The MTT-cobalt method gave a weak, even granular reaction in the nerve cells Formalin abolished the reaction totally

Isocitrate produced a similar reaction in the ganglion as it did with NAD

If PMS was used in the reaction mixture of NAD and NADP linked reductases a diffuse staining of the section resulted This was to be expected because many of the oxidative enzymes are soluble and could reduce tetrazolium salt via PMS in the incubating solution

Cytochrome Oxidase — The activity was observed in the cytoplasm of all ganglion cells localized in small round or oval granules (Fig 64) The number of granules varied in both small and large cells and their distribution was usually perinuclear especially in the small cells Nerve fibres exhibited a weaker granular reaction but in the capsular cells the granules were larger and more intensely reactive although fewer than in the ganglion cells Many thin myelinated fibres were strongly positive resembling those seen in the MAO preparations

Monoamine Oxidase — The activity as demonstrated with the tetrazolium reduction method of Glenner et al was seen in all ganglion cells (Figs 65 and 85) The cytoplasm was diffusely purplish but contained deep stained coarse granules The density and distribution of these granules varied considerably in individual cells A perinuclear concentration of the granules was mostly noticed but sometimes especially in large neurones they were evenly distributed throughout the cytoplasm (Fig 102) The nuclei were unstained The capsular and Schwann cells were negative Pre and postganglionic nerve fibres were both moderately stained, exhibiting both diffuse and granular staining In many thin apparently preganglionic fibres the myelin sheath showed an intense colour Positive fibres were seen in the postganglionic nerve trunks also These may well be fibres passing through the ganglion from the preganglionic trunk to the postganglionic ones (Fig 108)

To check whether this staining was non specific and due to the lipids of the myelin sheath the following experiments were carried out Sections cut from the ganglion were first preincubated in Marsilid solution Thereafter the sections were partly covered with a fresh liver section and incubated with it in an ordinary reaction mixture Only in those myelinated nerve fibres which were very close to the liver section were formazan granules seen but the intensity was clearly weaker than in normally incubated sections the others were totally negative This indicates that MAO activity in myelinated fibres cannot be due to the diffusion and lipid solubility of formazan When the sections were first incubated in NBT solutions and after careful rinsing put in a reducing agent e g $(NH_4)_2S$ solution, the nerve cells showed a strong formazan precipitate but the myelin sheaths were uncoloured This proves that NBT can become bound only to proteins but not to lipids Both of these results are thought to

support the idea that the positive reaction in myelinated nerve fibres is due to enzymatic activity

The hydrazone precipitation technique of Koelle and Valk revealed an identical distribution of MAO activity in the superior cervical ganglion (Fig 66)

PREGANGLIONIC DENERVATION

Histochemical Observations

Division of the sympathetic trunk had no effect on the activity or distribution of any oxidative enzymes in the superior cervical ganglion of the rat

POSTGANGLIONIC DENERVATION

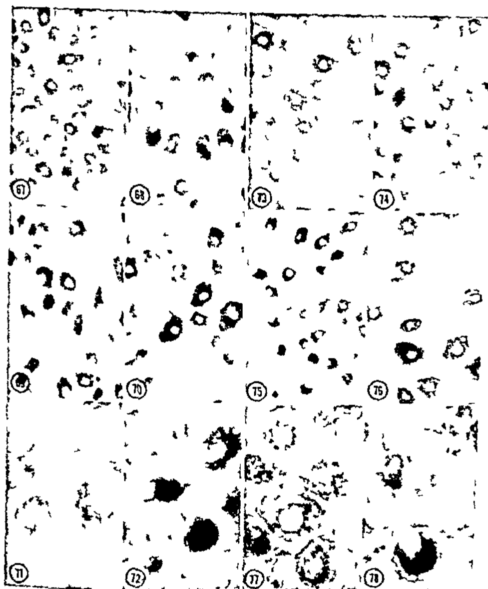
Histochemical Observations

During the first 12 hours following axotomy no changes in enzymatic activities were found On and after the first postoperative day, there was a progressive increase in the activity of NADH tetrazolium reductase and of all NAD linked tetrazolium reductases The enzymatic activities reached their maximum on the 3rd to 5th postoperative day and remained high for 30—40 days The increase was most pronounced in the NADH tetrazolium reductase activity (Figs 71 and 72) followed in order of less increased activity, by lactate (Figs 67—70) β-hydroxybutyrate and malate tetrazolium reductases (Figs 77 and 78) Changes in the activity of NADPH tetrazolium reductase were smaller than those in the activity of NADH tetrazolium reductase However glucose 6 phosphate (Figs 73—76) and isocitrate tetrazolium reductases showed a marked increase of enzymic activity After 100—150 days, large hyperactive ganglion cells were often observed (Figs 70 76 and 84)

A shift was observed in the cytoplasmic location of all oxidative enzymes during chromatolysis the activity was concentrated in a central region of the cell apparently vacated by the nucleus which had adopted an eccentric position (Figs 72 and 78) The Nissl substance was lacking from this central area as was seen in the neighbouring sections stained with gallocyanin (De Boer and Sarnaker 1956) or methyl green and pyronine (Kurnick 1955) In the denervated cells the formazan granules were larger and more intensely stained than those in the intact nerve cells of the contralateral ganglion The diffusely distributed reaction in the cytoplasm was also stronger than that in the control sections

The increase in the size of the mitochondria and in the intensity of the enzyme reaction was best demonstrated with succinate or α glycerophosphate and menadione (Figs 79 84) On the other hand, succinate tetrazolium reductase activity without PMS was only slightly increased in chromatolytic nerve cells

Monoamine oxidase activity (Figs 85—90) remained normal or somewhat raised during the first day Thereafter the activity decreased considerably in the chromatolytic nerve cells and especially in the nerve fibres being at its lowest from the 7th to the 30th 40th postoperative day This loss of reaction intensity was clearly demonstrated both with Glenner's and with Koelle and Valk's method



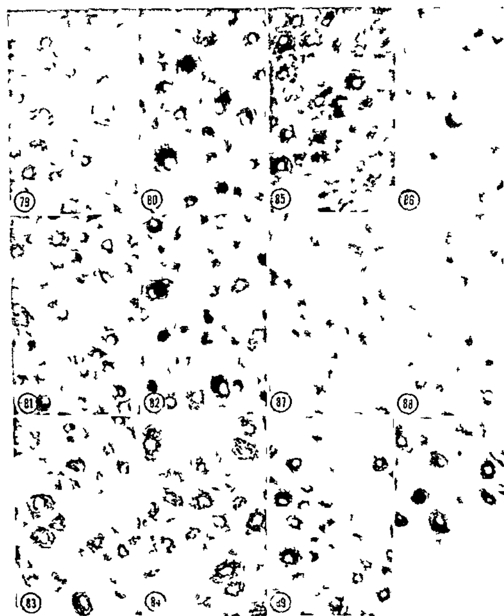
The effect of postganglionic denervation on oxidative enzyme activities

Figs 67—70 Lactate-tetrazolium reductase activity NBT fresh frozen sections x 175 *Fig 67* Control ganglion. *Fig 68* 1½ days after axotomy *Fig 69* 7 days after axotomy *Fig 70* 150 days after axotomy

Fig 71 NADH tetrazolium reductase activity NBT fresh frozen sections Control ganglion. x 470 *Fig 72* As *Fig 71* 8 days after axotomy

Fig 73—76 Glucose-6-phosphate tetrazolium reductase activity NBT fresh frozen sections x 175 *Fig 73* Control ganglion. *Fig 74* 1½ days after axotomy *Fig 75* 7 days after axotomy *Fig 76* 150 days after axotomy

Fig 77 Malate tetrazolium reductase activity NBT fresh frozen section. Control ganglion. x 470 *Fig 78* As *Fig 77* 8 days after axotomy



The effect of postganglionic denervation on oxidative enzym

Figs 79—84 α Glyceroph sphate menadione tetrazolium reductase activ zen sections x 175 *Fig 79* Control ganglion *Fig 80* 1 ½ days after days after axotomy *Fig 82* 7 days after axotomy *Fig 83* 50 days after a days after axotomy

Figs 85—90 Monoamine oxidase activity (Glenner et al) fresh frozen se Control ganglion *Fig 86* 1 ½ days after axotomy *Fig 87* 3 days after days after axotomy *Fig 89* 50 days after axotomy *Fig 90* 150 days aft

Fig 88

Cytochrome oxidase activity was somewhat decreased in ganglion cells from the 2nd postoperative day This weakening of activity was due to a decrease in the intensity of positive granules in the cytoplasm of the chromatolytic nerve cells On the other hand the remaining granules were larger than on the control side The interstitial structures also showed a clearly weakened activity

In the distal end of the axon stump an accumulation of MAO activity was observed (Fig 97) while the other oxidative enzyme activities were evenly distributed along the proximal axon stump (Fig 98)

Quantitative Determinations

On the basis of the above histochemical and electron microscopic studies the greatest changes in the histochemically demonstrable enzyme activities and mitochondrial morphology occurred between the first and third weeks after postganglionic denervation The quantitative enzyme determinations were there fore performed between the 7th and 13th postoperative days The results and statistical analyses are expressed in the same way as in quantitative determination of esterases

LD (μM NAD × 10-/mg protein/min)

Control y = 0 93 x
Axotomy y = 1 29 x
Total y = 1 21 x

Analysis of variance

Group	Sums of squares			f	s_0^2	F	$P<$
	Q	Q_R	Q_0				
Control	7424	7142	282	7	40 3	9 93	0 01
Axotomy	44902	42804	2098	21	99 9		
Total	52326	49102	3224	29	111 2		

MD (μM NAD × 10 /mg protein/min)

Control y = 0 93 x
Axotomy y = 0 75 x
Total y = 0 76 x

Analysis of variance

Group	Sums of squares			f	s_0^2	F	$P<$
	Q	Q_R	Q_0				
Control	11732	11010	722	7	103 1	3 58	
Axotomy	83219	81461	1758	21	83 6		
Total	94951	92172	2779	29	95 8		

G 6PD (μM NADP × 10^3/mg protein/min)

Control y = 0.97 x
Axotomy y = 1.63 x
Total y = 1.48 x

Analysis of variance

Group	Sums of squares			f	s_0^2	F	$P<$
	Q	Q_R	Q_0				
Control	3493	3117	376	7	53.9	10.79	0.01
Axotomy	32228	30238	1990	17	117.1		
Total	35721	32291	3430	25	137.2		

RED (μM cyt c × 10^3/mg protein/min)

Control y = 0.74 x
Axotomy y = 0.95 x
Total y = 0.86 x

Analysis of variance

Group	Sums of squares			f	s_0^2	F	$P<$
	Q	Q_R	Q_0				
Control	23254	19745	3509	7	501.3	3.22	
Axotomy	50277	46091	4186	19	220.3		
Total	73531	64883	8648	27	320.3		

Axotomy caused respectively the following percentage increases as judged from the left right difference in the original enzyme activity LD 29 % G 6 PD 63 % MD and RED activities did not change significantly after operation

DISCUSSION

Intracellular Localization of Oxidative Enzyme Activities

Because of the widespread distribution of mediating flavoprotein in the ganglion cells it was possible in the present study to demonstrate NAD and NADP linked tetrazolium reductase activities even at the intracellular level β Hydroxybutyrate mainly produced a formazan deposit in granules whereas lactate malate and glucose 6 phosphate tetrazolium reductase activities appeared both as a homogeneous and as a granular staining A localization exclusively in cytoplasmic granules could be observed only when succinate or α glycerophosphate menadione tetrazolium reductase were demonstrated With other substrates there was always a variable amount of diffuse background staining of the cells even when tissue blocks were fixed in cold formalin before freezing and cutting The reaction in granules probably indicates mitochondrial localization of the enzyme and homogeneous staining a cytoplasmic distribution Thus the observed intracellular distribution of these enzymes is principally in agreement with the already generally accepted biochemical observations that the glycolytic enzymes are found outside particles whereas the tightly bound particulate enzymes of the cytochrome system and TCA cycle as well as β hydroxybutyrate dehydrogenase are associated with mitochondria (see for ref Neilands and Stumpf 1958 Wakil 1963) Malate dehydrogenase however has been found to be present in easily extractable form in the cytoplasm also (Delbruck et al 1959) The observations of the present study are different from those of Walker (1963) and do not confirm his hypothesis that the mitochondria represent the exclusive site of activity of dehydrogenases and diaphorases

Novikoff (1960) has stated that NADH tetrazolium reductase activity is located both in mitochondria and in ergastoplasm in nerve cells while with NADPH as substrate only mitochondria can be seen The present observation of homogeneous staining of ganglion cells with NADPH is in agreement with the statement of Strecker and Giuditta (1959) that cerebral microsomes possess TPNH cytochrome c reductase activity

According to biochemical data both cytochrome and amine oxidase are considered to be associated with mitochondria (Schneider 1946 Schneider and Hoogeboom 1950 Blaschko et al 1957) although the microsomal fraction of the rat liver also contains some amine oxidase activity (Hawkins 1952) Fortunately both of these oxidases are structurally bound enzymes and therefore more suitable for demonstration in unfixed sections than the soluble dehydrogenases In the present study the Burstone technique for cytochrome oxidase and both techniques used for demonstration of MAO activity revealed mainly granular localization with only very weak background staining The distribution and the number of granules inside the neurones was similar in the two reactions

Tewari and Bourne (1962a b) have claimed that there is a correlation between the position of the nucleolus and the mitochondrial enzymes in the spinal ganglion neurones of the rat No evidence of such a correlation was obtained in the present study in the superior cervical ganglion of this species The histochemically demonstrated enzyme activity in the cytoplasm was situated either perinuclearly or else evenly throughout the cytoplasm without relation to the position of the nucleolus

Mitochondrial Heterogeneity

The reductase activity towards succinate obtained without artificial electron acceptors such as menadione seemed to be relatively weak and the number of stained granules was smaller than in the two oxidase reactions In the oxidase reactions the cytoplasmic granules showed a perinuclear concentration in most cells while in the succinate reaction they were usually scattered throughout the cytoplasm of the nerve cells

This observation might indicate the existence of different types of mitochondria in the sympathetic ganglion cell The mitochondria around the nucleus might be able to oxidize monoamines and cytochrome c but not succinate at least in histochemical systems

A similar observation on mitochondrial heterogeneity has also been made by Pearse et al. (1958) in micro-organisms In the present study all the mitochondria in the sympathetic ganglion cells were morphologically similar as judged from electron micrographs

Biochemical Aspects

In biochemical studies an enzyme capable of oxidizing α glycerophosphate without pyridine nucleotides was found in brain homogenates and proved to be insoluble (Green 1936) This enzyme was termed by Wosilait and Nason (1954) »α glycerophosphate menadione reductase» Hess and Pearse (1961) have since shown that α glycerophosphate menadione tetrazolium reductase activity is also bound to the mitochondria in the rat brain Histochemically this type of enzyme activity is usually present in very small amounts in the nerve cells except in those of the cornu Ammonis (Thomas and Pearse 1961) and in the spinal ganglion cells (Hess and Pearse 1961) but is highly active in the glial cells The superior cervical ganglion cells of the rat differ in this respect from most nerve cells because they showed high activity while the capsular cells were much more weakly positive

It is suggested that dihydroxyacetone phosphate produced by α glycerophosphate dehydrogenase activity could enter the Embden Meyerhof pathway at the level of triosephosphate isomerase (Pearse 1961) The high activity of this enzyme and that of the lactate tetrazolium reductase may thus indicate a potentially high glycolytic activity in the sympathetic ganglion cells

Malate (NAD linked) isocitrate (NAD and NADP linked) and succinate tetrazolium reductases indicate the activity of the Krebs cycle and they were all present in the nerve cells On the other hand the succinate tetrazolium reductase activity was weak and could only be adequately demonstrated after a prolonged incubation time The low activity of this enzyme would at first sight seem to be a rate limiting factor in the citric acid cycle of the sympathetic ganglion cells Since PMS and menadione presumably obtain electrons directly from succinate dehydrogenase it is possible to demonstrate the activity of this enzyme directly It therefore seems that electron transfer from succinate dehydrogenase to tetrazolium is governed by the co-factors involved rather than by the rate of the initial reaction

Enzyme Activities in Nerve Cells and Glial Cells

In the capsular and Schwann cells oxidative enzyme activities were observed in the present study to be predominantly perinuclear and in the form of small intensely blue granules It is probable that NADH and NADPH tetrazolium reductase activities are located only in the mitochondria of these cells and that therefore all other pyridine nucleotide dependent enzymes have a similar localization It was somewhat difficult to compare the activity of these cells with that of the neurones because of the difference in the distribution of the reaction However all the pyridine nucleotide linked enzymes seemed to be less active in the glial cells than in the nerve cell bodies On the other hand succinate tetrazolium reductase and cytochrome oxidase activities were equal or higher respectively in the capsular cells A low level of oxidative metabolism in the neuroglia has also been noticed in many biochemical observations (Pope et al 1956 Lowry et al 1954 Robins and Smith 1953) Hyden and Pigon (1960) on the other hand found cytochrome oxidase and succinic oxidase activity to be much higher in the oligodendroglia than in an equal volume of nerve cell from Deiters nucleus

Qualitative histochemistry with the older tetrazolium salts rather consistently failed to demonstrate any considerable amounts of oxidative enzyme activity in the neuroglia as compared with the neurones (Padykula 1952 Mustakallio 1954) Studies using MTT have confirmed the low level of oxidative enzyme activity in the glial tissue (Thomas and Pearse 1961 Felgenhauer and Stammler 1962) However when NBT has been used as final electron acceptor different results have been obtained (Potanos et al 1959 Romanuel and Cohen 1960 Becker et al 1960 Friede 1961 Rubinstein et al 1962 Friede et al 1963 Kumamoto and Bourne 1963) Thomas and Pearse (1961) and Pearse and Hess (1961) have suggested that the intense staining of glial cells with NBT depends on the »substantivity» of this tetrazolium salt but also on the so-called »nothing dehydrogenase» reaction (Zimmermann and Pearse 1959) The positive reaction in glial cells seen in the present study cannot be an artifact for the above mentioned reasons since the reactions were negative with all the three tetrazolium salts used if the incubation was carried out without substrate or the sections were inactivated with boiling water Furthermore Hitzeman (1963) observed that after lipid extraction with cold acetone there was no unspecific absorption of NBT in testicular tissue and the »nothing dehydrogenase» reaction was absent

Axon Reaction

In the present study a definite increase in most of the histochemically demonstrated dehydrogenase activities was observed in the chromatolytic sympathetic ganglion cells and the intracellular distribution was also changed This is in agreement with the observation of Klein (1960) who reported redistribution and increase in succinate dehydrogenase activity in spinal ganglion cells and that of Kreutzberg (1963) who found a marked increase of TPN diaphorase and TPN linked dehydrogenase activities in motor nerve cells during chromatolysis

Friede (1959d) on the other hand found that chromatolysis was accompanied by a depletion of succinate dehydrogenase TPN and DPN diaphorase in the nerve cell body and an accumulation of these enzymes at the site of the interruption A loss of succinate dehydrogenase and DPN diaphorase was also observed by Kumamoto and Bourne (1963) and a biochemically measured loss of cytochrome oxidase and succinate dehydrogenase activities by Howe and Mellors (1945) and Howe and Flexner (1947) Concerning cytochrome oxidase the results presented in the present report are in agreement with the biochemists view but the histochemically demonstrable succinate dehydrogenase activity seemed to increase

The chemical estimation of malate dehydrogenase and NADH cytochrome c reductase activity in the ganglion in the present work indicated no change due to the operation whereas the activities of glucose 6 phosphate and lactate dehydrogenase which are both purely soluble enzymes were increased when estimated histochemically or chemically Histochemically the NADH and malate tetrazolium reductase reactions were found to be stronger in the ganglion cells on the operated side than on the control side Thus the histochemical observations and biochemical enzyme assays diverged from each other in some respects

The increased formazan reduction in chromatolytic nerve cells may be due to the increased permeability of the mitochondrial membrane to the substrates and the tetrazolium salt In conditions of magnesium deprivation (Hess et al 1959) hypervitaminosis D (Scarpelli et al 1960) and dehydration of the organism (Niemi et al. 1960) mitochondrial swelling and an increase of the formazan deposit in the mitochondria has been noticed in the kidney tubules when succinate dehydrogenase and DPN-diaphorase are histochemically demonstrated Similar observations have been made by Portugalov et al (1962) in nerve cells after intense stimulation of the peripheral nerve fibres Chemical estimation of the same enzymes and cytochrome oxidase showed a decreased activity however This discrepancy was attributed to accumulation of reduced thiol groups in the nerve cells which led to swelling of the mitochondria and a greater permeability of their membrane to substrate and tetrazolium salt (Lehninger and Schneider 1959)

The electron microscopic observations of the present study revealed indeed a considerable swelling of mitochondria during chromatolysis This was most pronounced between the 7th and 15th postoperative days

Chemical measurement and histochemical demonstration of glucose 6 phosphate dehydrogenase indicated increased activity of the pentose phosphate cycle during chromatolysis This metabolic cycle has been suggested to function in biosynthetic processes (Dickens 1955) perhaps by increasing the availability of reduced NADP (Wilson and Siperstein 1959) or providing pentose intermediates for the synthesis of ribonucleic acid (Dickens 1955 Glock and MacLean 1954 1955) Chemical determinations indeed have shown that efficient production of RNA proteins and lipids occurs during nerve cell regeneration (Brattgård et al 1957) Studies using labelled methionine and orotic acid confirm this observation (Fisher et al. 1958 Brattgård and Hyden cited by Hydén 1960)

IV CATECHOLAMINES IN THE SUPERIOR CERVICAL GANGLION

EARLIER INVESTIGATIONS

The sympathetic ganglia are known to contain catecholamines principally noradrenaline (Euler 1947 Vogt 1954) Eranko (1952, 1955) was the first to describe a method for histochemical demonstration of noradrenaline in adrenomedullary cells with formalin induced fluorescence The method was rendered more sensitive when it was observed that an intense fluorescence developed in catecholamine-containing cells when freeze-dried sections were exposed to formaldehyde vapour (Eranko 1961 Falck and Torp 1961) Falck et al (1962) studied the nature of the reaction and concluded that fluorescence was given only by aromatic primary or secondary amines having hydroxyl groups at the 3 and 4 positions (e g dopamine norad renaline and adrenaline) The secondary amines were less fluorescent however and needed longer exposure to formalin vapour than the primary amines

Recently Eranko and Harkonen (1963) have described the distribution of noradrenaline in the ganglion cells of the superior cervical ganglion of the rat as revealed by the formalin induced fluorescence method. Soon thereafter Hamberger and his co-workers (Hamberger and Norberg 1963 Hamberger et al. 1963) published their independent studies on fluorescent monoamines in the sympathetic ganglia of the rat and cat after administration of various drugs and after preganglionic denervation.

The effect of postganglionic denervation on the catecholamines in the sympathetic ganglion (Eranko and Harkonen 1964b) and in the spinal cord (Dahlstrom and Fuxe 1964) has been briefly described

METHODS

Fresh ganglia were quickly removed and frozen with the aid of metal disc forceps precooled in liquid air (Eranko 1954) The ganglia were first dried in vacuo at —35 C for 4 days and then treated with formaldehyde vapour at +80 C for 1 hour in a closed Petri dish containing para formaldehyde Thereafter the specimens were embedded in paraffin in vacuo at +56 C Sections were cut at 10 μ and placed on slides deparaffinized with benzene and mounted in Entellan (Merck)

The fluorescence was studied with an Osram HBO 200 high pressure mercury lamp as light source The light was filtered through two Schott K2 heat-absorbing filters and Schott BG12 and BG23 filters Above the non fluorescent objectives Schott OG1 and Leitz Euphos filters were inserted to cut out the ultraviolet and blue light Occasionally a dark field condenser was used for photography of the sections

RESULTS

NORMAL GANGLION

A green fluorescence was observed in the perikaryon of all the ganglion cells but not in the nuclei (Fig 91) Its intensity varied considerably in individual cells Usually it appeared diffusely throughout the cytoplasm, but in many cells strongly fluorescent clusters could be seen in the cytoplasm and in the nerve fibres between the cells The fluorescent material was very sensitive to humidity and disappeared totally if the sections were treated with water or even breathed on In such sections only orange fluorescent granules could be seen probably showing the localization of lipofuscin pigment or flavoproteins in the mitochondria This type of fluorescence could easily be distinguished by visual examination from the green amine fluorescence

Occasionally groups of small oval cells exhibiting very brilliant yellow fluorescence were seen in a ganglion especially in the nerve bundles and sometimes outside the ganglion This fluorescence was water resistant These cells are apparently not nerve cells or chromaffin cells because ganglia fixed in potassium dichromate did not show chromaffin cells of this type

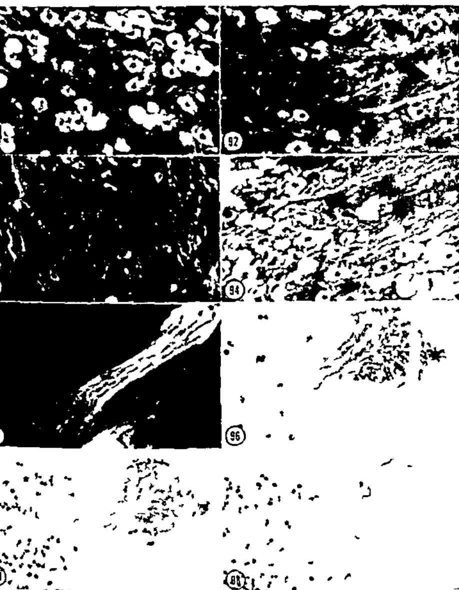
The ganglia of animals whose catecholamine stores had been depleted with reserpine (5 mg/kg daily three injections) did not show any specific fluorescence

PREGANGLIONIC DENERVATION

Preganglionic denervation had no significant effect on the formaldehyde induced fluorescence of the superior cervical ganglion

POSTGANGLIONIC DENERVATION

During the first 12 hours after transection of the postganglionic nerve fibres no marked changes in the intensity of fluorescence were found On the second postoperative day the intensity was clearly decreased in most of the neurones (Fig 92) At this time a very strong fluorescence of an intensity clearly exceeding that of normal postganglionic fibres was observed in the distal ends of the axon stumps (Fig 95) Between 7 and 20 days after the operation the chromatolytic ganglion cells showed hardly any specific fluorescence only at the periphery of some cells could a weak fluorescent circle be seen (Fig 93) In the ganglia taken 50—150 days after section brilliantly fluorescent nerve cells could again be seen (Fig 94) However the number of fluorescent nerve cells was smaller than in the control ganglia



r 91—94 The effect of postganglionic denervation on catecholamines Freeze-dried pre
ations x 175

: 91 Control ganglion

: 93 10 days after axotomy

Fig 92 2 days after axotomy

Fig 94 90 days after axotomy

r 95—97 Accumulation of catecholamines AChE and MAO activities in the distal end
the proximal axon stump

: 95 Catecholamine fluorescence x 25

: 97 MAO (Glenner et al) x 37

Fig 96 AChE x 37

Fig 98 Succinate tetrazolium reductase (PMS) NBT No accumulation of enzyme activity in the distal end of the proximal axon stump x 37

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V CORRELATION BETWEEN CARBOXYLIC ESTERASES, OXIDATIVE ENZYMES AND CATECHOLAMINES IN THE SYMPATHETIC GANGLION CELLS

EARLIER INVESTIGATIONS

Tewari and Bourne (1962a) observed a wide range in the intensity and distribution of several histochemically demonstrable enzyme reactions in the spinal ganglion cells of the rat and suggested that the spinal ganglion cells undergo a continuous metabolic cycle However they did not study the different enzyme reactions in the same cell

Fredricsson and Sjoqvist (1960 1962) compared the distribution of AChE and Nissl substance in the same sympathetic ganglion cells using neighbouring sections but did not observe any correlation between these two reactions Reciprocal relations between AChE and fluorescent monoamines were observed recently by Hamberger et al (1963) who studied these substances in adjacent cryostat sections of various sympathetic ganglia of the cat However Eranko and Harkonen (1964a) using the same section for demonstration of catecholamines and AChE in the superior cervical ganglion of the rat did not find any direct or inverse correlation between the intensity of fluorescence and the AChE activity both being high in many nerve cells

Great variation in the histochemically demonstrated enzyme activities and fluorescent monoamines among the individual sympathetic nerve cells may be due either to cyclic changes in essentially similar cells or to the presence of cells that are permanently entirely different e g cholinergic To study this problem it was decided to compare the different reactions in individual cells

METHODS

Most of the enzyme reactions were compared in thin adjacent cryostat sections in which the same ganglion cells could be found Sometimes it was possible to compare the reactions in the same sections e g NADH tetrazolium reductase and ns E reactions The section was first incubated in the former reaction solution registered by microphotography and thereafter incubated with the latter substrate solution

Formalin resistant enzymes e g ChEs and fluorescent monoamines were compared in the same section as described by Eranko (1964) After freeze-drying the ganglia were treated with formaldehyde vapour at 50—55 C for one hour and embedded in paraffin wax under a vacuum The fluorescence due to noradrenaline was recorded by microphotography after deparaffini

DISCUSSION

Since the fluorescence developed relatively rapidly in the ganglion its colour was green and it was totally absent in reserpine treated animals there seems little doubt that the fluorescent material in the sympathetic ganglion of the rat is noradrenaline (see Falck et al. 1962, Eranko and Harkönen 1963 Eranko 1964)

The noradrenaline content varied greatly in individual cells It may be that some of the nerve cells in the sympathetic ganglion are »more cholinergic» in nature and contain smaller amounts of catecholamines On the other hand the individual cells might be in different functional or metabolic states Because preganglionic denervation did not diminish the variation in the intensity of fluorescence among the ganglion cells it is improbable that different functional states are induced by mediation of the preganglionic fibres from autonomic centres in the central nervous system The depletion of the fluorescent material from the cell body and its accumulation in the terminal part of the proximal stump after section of the nerve fibre suggests that catecholamines are manufactured and stored in the nerve cell cytoplasm and transported along the axon to the nerve terminals (see Eränkö and Härkönen 1963) In this case the strong fluorescence observed in some ganglion cells would indicate in fact increased catecholamine synthesis and accelerated metabolism

As mentioned earlier monoamine oxidase and acetylcholinesterase activity were similarly accumulated in the sectioned nerve stump and depleted in the nerve cell body After section of the axon the cell body probably ceases to synthesize the transmitter and the enzymes connected primarily with nerve function and intensifies the synthesis of structural proteins and the enzymes involved in their synthesis

The observation of diffuse cytoplasmic fluorescence and granular fluorescence suggests that in sympathetic ganglion cells and nerve fibres noradrenaline is present partly in soluble form and partly bound to granules (Eranko and Harkonen 1963) which is in agreement with earlier observations made using biochemical methods (Euler and Hillarp 1956)

V CORRELATION BETWEEN CARBOXYLIC ESTERASES, OXIDATIVE ENZYMES AND CATECHOLAMINES IN THE SYMPATHETIC GANGLION CELLS

EARLIER INVESTIGATIONS

Tewari and Bourne (1962a) observed a wide range in the intensity and distribution of several histochemically demonstrable enzyme reactions in the spinal ganglion cells of the rat and suggested that the spinal ganglion cells undergo a continuous metabolic cycle However they did not study the different enzyme reactions in the same cell

Fredricsson and Sjoqvist (1960 1962) compared the distribution of AChE and Nissl substance in the same sympathetic ganglion cells using neighbouring sections but did not observe any correlation between these two reactions. Reciprocal relations between AChE and fluorescent monoamines were observed recently by Hamberger et al. (1963) who studied these substances in adjacent cryostat sections of various sympathetic ganglia of the cat However Eranko and Harkonen (1964a) using the same section for demonstration of catecholamines and AChE in the superior cervical ganglion of the rat did not find any direct or inverse correlation between the intensity of fluorescence and the AChE activity both being high in many nerve cells

Great variation in the histochemically demonstrated enzyme activities and fluorescent monoamines among the individual sympathetic nerve cells may be due either to cyclic changes in essentially similar cells or to the presence of cells that are permanently entirely different e g cholinergic To study this problem it was decided to compare the different reactions in individual cells

METHODS

Most of the enzyme reactions were compared in thin adjacent cryostat sections in which the same ganglion cells could be found Sometimes it was possible to compare the reactions in the same sections e g NADH tetrazolium reductase and ns E reactions The section was first incubated in the former reaction solution registered by microphotography and thereafter incubated with the latter substrate solution

Formalin resistant enzymes e g ChEs and fluorescent monoamines were compared in the same section as described by Eranko (1964) After freeze-drying the ganglia were treated with formaldehyde vapour at 50—55 C for one hour and embedded in paraffin wax under a vacuum. The fluorescence due to noradrenaline was recorded by microphotography after deparaffini

zation of the section with xylene Thereafter the section was allowed to dry and was incubated in substrate solution The incubation time for ChEs was usually 6 hours and with α naphthyl acetate the reaction took 10 minutes

After freeze-drying some ganglia were embedded directly in paraffin wax and neighbouring sections were used for demonstration of the enzyme and the fluorescence Formaldehyde treatment of dried cryostat sections was sometimes used for the study of monoamines (Hamberger and Norberg 1964) whose distribution could then be compared with the histochemically demonstrated enzyme activity in the same or neighbouring section

RESULTS

Oxidative Enzymes

When different histochemically demonstrable dehydrogenases were compared with each other it was observed that generally a positive correlation between them occurred. However depending on the reaction some variations were observed In Figs 99 and 100 NADH tetrazolium reductase and succinate tetrazolium reductase in neighbouring sections are compared The former reaction is homogeneous and the latter particulate However there is a good correlation between these two reactions

When the dehydrogenase reactions were compared with MAO activity it was observed that these reactions often had similar levels of activity in the same individual cells (Figs 101 and 102) However because the large nerve cells were often weakly MAO positive but showed moderate dehydrogenase reactions the differences between individual cells in the MAO preparations were greater than those in the dehydrogenase reactions

Carboxylic Esterases

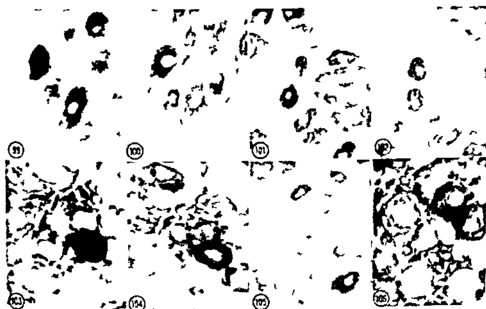
Only a few ganglion cells were strongly ns ChE-positive the remainder being negative or weakly positive The strongly ns ChE-positive nerve cells were also regularly strongly AChE positive (Figs 103 and 104) However many strongly AChE positive cells were weakly ns ChE-positive or even negative because the number of strongly AChE-positive ganglion cells was clearly greater than that of ns ChE positive cells

No correlation between I s ns I activity and either of the ChE activities could be observed (Figs 105 and 106)

Oxidative Enzymes and Carboxylic Esterases

No correlation between histochemically demonstrable dehydrogenases and carboxylic esterases could be observed

The strongly ns ChE positive cells were invariably weaker in MAO activity than the others (Figs 107 and 108) A similar inverse correlation was also ob



Distribution of enzyme activities in the same ganglion cells Fresh frozen sections x 255

Fig 99 NADH tetrazolium reductase

Fig 100 Succinate tetrazolium reductase in the neighbouring section

Fig 101 NADPH tetrazolium reductase

Fig 102 MAO in the neighbouring section.

Fig 103 NsChE

Fig 104 AChE in the neighbouring section

Fig 105 NsE.

Fig 106 NsChE in the neighbouring section

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served in most of the strongly AChE positive ganglion cells However in the remaining cells the MAO activity varied without any correlation to the AChE content of the cells

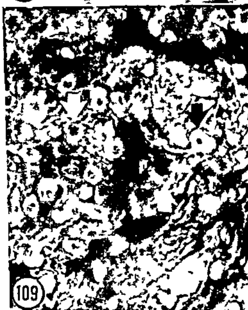
Catecholamines and Carboxylic Esterases

When formalin induced fluorescence and AChE activity were compared in the same section no correlation could be noted The strongly fluorescent cells exhibited a strong moderate or weak AChE activity and the strongly AChE positive cells showed a brilliant moderate or weak fluorescence

However when total esterase activity was demonstrated with α-naphthyl acetate as substrate the strongly fluorescent nerve cells usually exhibited a weaker esterase reaction than the weakly fluorescent ones and the strongly esterase positive cells fluoresced weakly However exceptions to this inverse correlation could be noticed in some cells The strongly NsChE positive nerve cells always showed very weak fluorescence (Figs 109 and 110)







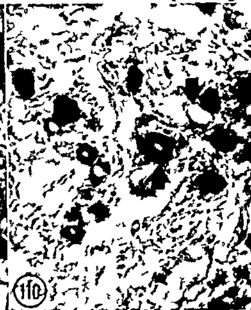


Fig 107 Ns ChE 510

Fig 108 MAO in the neighbouring section x 510

Fig 109 Catecholamine fluorescence Fresh frozen freeze-dried section treated with formaldehyde vapour x 220

Fig 110 The same section stained for ns ChE x 220

DISCUSSION

On the basis of several experimental observations Burn and Rand (1960 1962) proposed the idea of a cholinergic mechanism for the release of noradrenaline by postganglionic adrenergic fibres (see the section on carboxylic esterases p 45) The present observations lend further support to this idea because all ganglion cells exhibited AChE activity without regard to high or low noradren aline content This is not the case in the sympathetic ganglia of the cat, in which some cells apparently cholinergic ones contain no noradrenaline but exhibit an intense AChE activity, while others apparently adrenergic ones show hardly any AChE activity but contain high concentrations of noradrenaline (Hamber ger et al 1963) In the rat in which no such sharp distinction between adrenergic and cholinergic cells can be made it is possible that the cells exhibiting a strong ns ChE activity but little fluorescence might be considered »more cholinergic» than the others

The inverse correlation between the intensity of fluorescence and the total esterase activity in ganglion cells is also probably due to the ns ChE activity demonstrable with α naphthyl acetate after formaldehyde treatment

Increased oxidative enzyme activities are known to indicate increased energy production Because the histochemically demonstrable dehydrogenase activities seem to correlate well with each other in ganglion cells it appears that they operate together and cyclic changes occur in these cells The function of ns Es however is apparently not closely connected with that of the oxidative enzymes

VI COMMENTS ON THE HISTOCHEMISTRY OF THE AXON REACTION

It is not within the scope of the present paper to attempt a full examination of the axon reaction The interested reader is referred to the excellent recent review by Brodal (1964) However the results of the present work make it possible to throw some additional light on the changes occurring during the axon reaction and may invite some speculation as to the functional implications of these changes

Examination of the histochemical changes observed is facilitated by dividing the axon reaction into four stages (see Fig 111)

1 *The Initial Stage* (0—2 days) During the first 12 hours no changes in axotomized ganglia could be seen Thereafter the ganglion cells became swollen and the Nissl substance gradually disappeared from the central area Concomitantly with these changes the AChE and ns ChE activities disappeared from the nerve cells in two days Ns E activity decreased somewhat during this time and the activity tended to be concentrated in the central area Catecholamine fluorescence was somewhat but not much weaker than in the control ganglia The activities of all the dehydrogenases CytO and MAO varying were increased tending to concentrate in the central region of the cell apparently vacated by the nucleus which had adopted an eccentric position

2 *The Maximal Stage* (2—30 days) The ganglion cells were no longer swollen and the Nissl substance could be seen only in the periphery of the nerve cells The activity of the oxidative enzymes and ns E was concentrated in a small central area of the ganglion cells The dehydrogenase activities were still clearly increased but the CytO MAO and ns E activities were decreased AChE and ns ChE reactions remained negative in the nerve cells and their intensity was now clearly dimished in the interstitial tissue also Catecholamine fluorescence was absent from most nerve cells

3 *The Recovery Stage* (30—100 days) This stage was characterized by the tendency of all the reactions to return towards normal While many ganglion cells indeed appeared normal others still resembled those in the previous phase Therefore there were great variations in the histochemical reactions of individual cells

4 *The Late Stage* (100—150 days) The number of nerve cells in the ganglion was apparently smaller than before the operation The size of the surviving cells was larger however All the histochemical reactions had a normal distribution but the enzyme activities were clearly higher than those in the control

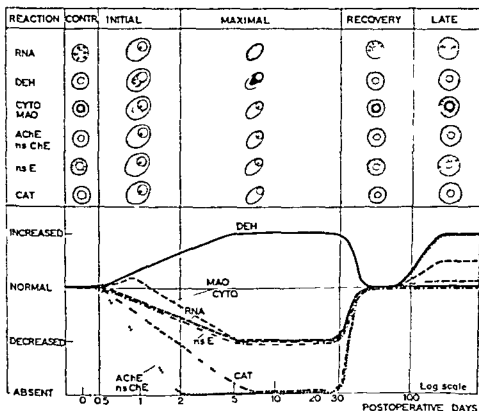


Fig 111 Diagramme schematically illustrating the changes in the distribution and intensity of various histochemical reactions during the axon reaction Changes in the size of the nerve cell are somewhat exaggerated. The curves are drawn to indicate the general tendency only Increase in the activities of MAO and CYTO 0—1 days after denervation refer to the perinuclear reaction which did not increase in all ganglion cells Changes in the other enzyme reactions were less subject to variation in individual cells Abbreviations RNA ribonucleic acid DEH dehydrogenase CYTO cytochrome oxidase MAO monoamine oxidase AChE acetylcholinesterase ns ChE, non-specific cholinesterase ns E non specific esterase CAT catecholamines

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ganglia except for the ns E and MAO activities which appeared normal The catecholamine fluorescence had returned to normal

From Fig 111 it is clear that the different histochemical reactions studied did not change in the same way during the different stages of chromatolysis Axotomy resulted in a strong increase in the activity of the oxidative enzymes perhaps so as to allow the nerve cell to obtain the extra energy needed for regenerative processes During the maximal stage the whole oxidative metabolism was concentrated in a small area close to the nucleus where active transfer of nuclear RNA to the cytoplasm is known to play an important role in protein

synthesis (Brachet 1961) Whether the oxidative enzyme demonstrated was involved in glycolytic TCA pentose phosphate or fatty acid metabolism and regardless of its normal intracellular distribution this phenomenon always occurred

On the other hand a loss of catecholamines MAO and ChEs was observed These either disappeared totally or decreased markedly in the ganglion cells accumulating in the distal end of the proximal axon stump This observation suggests that the transmitter substances and the enzymes involved in their inactivation are normally synthesized in the nerve cell bodies and transported along the axon to the nerve terminals where they function In the intact neurone synthesis is continuous but when contact with the effector organ is severed these substances lose their importance and the energy normally required for their synthesis is perhaps then used for protein synthesis in the interests of regeneration

The disappearance of catecholamines ChEs and MAO occurred asynchronously AChE activity disappeared entirely during the initial stage whereas the decrease in the catecholamine content was much slower only being completed after 7 days This observation indicates that ACh (provided that ACh and AChE are co-existent) disappears much earlier than catecholamines from adrenergic neurones during chromatolysis Such a difference suggests different types of function for ACh and catecholamines in the sympathetic ganglion cells

That the division of the axon also has an effect on the preganglionic fibres is shown by the present observation that after axon division these exhibited clearly diminished AChE activity This agrees with the observation of Brown (1958) of decreased AChE activity in the preganglionic fibres due to axotomy and with the observation that ganglionic transmission no longer occurs after division of postganglionic nerve fibres although the release of ACh is normal (Brown et al 1952 Brown and Pascoe 1954 McLennan 1954 and Dhar 1958) These observations could be taken to indicate that during chromatolysis the ACh receptors in the postganglionic nerve cell membrane lose their sensitivity towards ACh which through feedback affects the synthesis of ACh and AChE activity in the preganglionic fibres It is noteworthy that the axon reaction caused a loss of ns ChE from the capsular cells also This suggests that their normal function is dependent on the functional state of the nearby ganglion cell

It is apparent that ChEs and ns Es serve entirely different functions in the nerve cells because changes in their distribution and activity were dissimilar during chromatolysis Tentatively ns Es might perhaps be considered as catabolic enzymes in protein or lipid metabolism If this were so the decreased ns E activity after axotomy would explain the increase in the amount of proteins and lipids in the nerve cells which has actually been observed (Brattgård et al 1957)

In the recovery phase the axons of some of the ganglion cells had probably reached the effector organ making it understandable that their enzymic activity had returned to normal On the other hand those nerve cells which had not succeeded in contacting the periphery apparently died or if surviving were still engaged in regenerative processes as indicated by the large size and intense enzymic activity

The observations presented make it clear that the axon reaction is an even more complicated process than has as yet been realized and that it is composed of several apparently independent changes in the cellular function of the ganglion cells It is to be hoped that further studies will provide better insight into the nature of each individual process and subsequently into the integration of all factors participating in the axon reaction

SUMMARY

The present investigation deals with the normal distribution of carboxylic esterases oxidative enzymes and catecholamines in the superior cervical ganglion of the rat and the effect of pre and postganglionic nerve division

Ultrastructure (Chapter I) The observations on the ultrastructure of the normal ganglion were in agreement with earlier reports After axotomy the volume of individual mitochondria increased progressively reaching a maximum between the 7th and 15th days Two or three months after axotomy the ultrastructure of most ganglion cells had returned to normal.

Carboxylic Esterases (Chapter II) The carboxylic esterases were histochemically studied in fresh and in fixed sections as well as by starch gel electrophoresis using different substrates and as selective inhibitors 284C51 iso-OMPA eserine E600 and p-chloromercuribenzoate. With the aid of these the esterase activity was divided into four types acetylcholinesterase (AChE) non specific cholinesterase (ns ChE) E600-sensitive non specific esterase (E-s ns E) and E600-resistant non specific esterase (E r ns E)

AChE was histochemically demonstrable in all ganglion cells The intensity of the reaction ranged from weak to strong The preganglionic nerve fibres were more strongly stained than the postganglionic ones

The ns ChE activity was very strong in some ganglion cells which were also strongly AChE positive The remainder showed little or no ns ChE activity The interstitial tissue was strongly positive probably owing to the activity of the capsular and Schwann cells In zymograms three AChE fractions could be observed with acetylthiocholine and α naphthyl acetate as substrates while only one broad zone exhibited ns ChE activity

Preganglionic denervation abolished the AChE activity from the preganglionic fibres and the ns ChE activity was somewhat reduced in the capsular cells

Axotomy resulted in total disappearance of AChE and ns ChE from all the chromatolytic ganglion cells in two days At the same time, a strong AChE activity was observed in the distal ends of the axon stumps The interstitial tissue also showed a clearly decreased activity of both ChEs In the zymogram one of the AChE-active fractions was no longer visible the two others showed decreased activity On the 7th to 13th postoperative day the ChE activity on the denervated side, determined spectrophotometrically was 39 % of the activity on the control side. After 50 days many nerve cells were AChE positive again and between 100—150 days after axotomy they exhibited an activity clearly stronger than normal.

All ganglion cells exhibited a strong E s ns E activity in fresh sections This activity representing the desmo component of E s ns E, was largely inactivated by formalin fixation whereas similar esterase activity could be demonstrated only after formalin fixation in the interstitial tissue (lyo-component of E s ns E) Formalin sensitive and resistant esterases were in separate bands in the zymograms obtained by electrophoresis

All E-r ns Es activity was readily water soluble and it was demonstrable only after formalin fixation in the Schwann and capsular cells as well as in some ganglion cells In zymograms developed with α naphthyl acetate E r ns E activity appeared in five separate bands

By comparing the reactions obtained with different substrate inhibitor combinations in fresh and fixed sections as well as in zymograms it was possible to correlate a histochemical reaction in a section with a specific area in the zymogram Because not all the esterase bands showed the same substrate and inhibitor characteristics they cannot be regarded as isozymes

Preganglionic denervation had no effect on the ns E activity in the ganglion Axotomy caused reduction of E s ns E activity in the ganglion cells both histochemically and chemically (67 % of the activity on the control side on the 7th to 13th postoperative day) but it was never totally absent Histochemically demonstrable E-r ns Es did not show any changes but chemically the decrease was 14 % (on the 7th to 13th postoperative day) After 50 days recovery of ns Es was observed

Oxidative Enzymes (Chapter III) Most histochemically demonstrable oxidative enzymes (NADH NADPH α glycerophosphate lactate malate β hydroxy butyrate isocitrate glucose 6-phosphate and α glycerophosphate menadione tetrazolium reductases) but not succinate tetrazolium reductase or cytochrome oxidase were more active in the ganglion cells than in the surrounding capsular cells Monoamine oxidase cytochrome oxidase succinate β hydroxybutyrate and α glycerophosphate menadione tetrazolium reductase activities were mainly located in cytoplasmic granules while the others showed diffuse cytoplasmic staining as well

Preganglionic denervation had no effect on the oxidative enzyme activities After division of the axon all the oxidative enzymes were concentrated in a central area of the chromatolytic ganglion cells and the staining intensity was increased except for the cytochrome oxidase and monoamine oxidase preparations Fifty days after operation many nerve cells showed normal distribution and intensity After 100—150 days large hyperactive ganglion cells were often observed

Quantitative spectrophotometric determinations showed that glucose-6-phosphate and lactate dehydrogenase activities were 63 % and 29 % higher than those of the control ganglia between the 7th and 13th postoperative days Malate dehydrogenase and NADH-cytochrome c reductase activities did not change as a result of the operation

Catecholamines (Chapter IV) The sympathetic ganglion cells showed a weak moderate or strong green fluorescence due to catecholamines when freeze-dried ganglia were treated with dry formaldehyde vapour Preganglionic denervation had no effect on the content of catecholamines whereas division of postganglionic fibres caused depletion of catecholamines from nerve cell bodies within

7 days after division and their accumulation in the terminal part of the proximal stump After 50—150 days brilliantly fluorescent nerve cells could be seen again The number of these cells was smaller however than in the control ganglia

Correlation of Enzyme Activities and Catecholamines (Chapter V) The activities of the histochemically demonstrable carboxylic esterases oxidative enzymes and catecholamines were compared in the same sympathetic ganglion cells using the same section or neighbouring ones

The strongly ns ChE positive nerve cells were also regularly strongly AChE-positive but many strongly AChE-positive cells were weakly ns ChE-active or even negative, i e the number of AChE positive cells was greater than that of ns ChE-positive cells No correlation between AChE and catecholamines or E s ns Es and either of the ChEs could be observed. There was however an inverse correlation between the intensity of the ns ChE reaction and the con centration of fluorescent monoamines

No correlation between the activity of esterases and that of oxidative enzymes could be detected The strongly ns ChE-positive cells however were invariably weaker in MAO activity than the others Most oxidative enzymes showed the same degree of activity in the same ganglion cell.

Comments on the Histochemistry of the Axon Reaction (Chapter VI) On the basis of the histochemical changes observed the axon reaction can be divided into four stages initial maximal, recovery and late stages In the *initial stag* (0—2 days) all oxidative enzyme activities were increased and they tended to con centrate in the central area of the chromatolytic nerve cell. AChE and ns ChE activities disappeared from the nerve cells and the catecholamine content was but slightly decreased In the *maximal stage* (2—30 days) the oxidative enzyme ac tivities were located in a small central area of the ganglion cell cytoplasm and all the dehydrogenase reactions were increased. The cytochrome oxidase and monoamine oxidase activities were now decreased, however and the AChE and ns ChE reactions were negative in the nerve cells and now clearly decreased also in the interstitial tissue. Catecholamine fluorescence was absent. In the *recovery stage* (30—100 days) the histochemical reactions seemed to normalize In the *late stage* (100—150 days) the surviving ganglion cells were large and all the histochemical reactions had normal distributions but their activity was stronger than in the control ganglia The significance of the distribution and activities of enzymatic reactions and catecholamine content in these stages is discussed.

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THE SYMPATHETIC ADRENERGIC NEURON

Some Characteristics Revealed by Histochemical Studies on the Intraneuronal Distribution of the Transmitter

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I. INTRODUCTION

Thanks mainly to the fundamental work of VON EULER and his school it is now generally accepted that NA[1] is the transmitter of the sympathetic adrenergic system in mammals and that this amine is stored in high concentrations in specific nerve granules (see EULER 1959, 1961, 1962). During the last years there has developed an ever increasing interest in the physiology, biochemistry and pharmacology of the adrenergic innervation and various adrenergic mechanisms. The morphological basis for work in this field has sometimes been poor since earlier histological studies of the sympathetic nervous system were often invalidated by the lack of specificity in the methods used (cf. HILLARP 1959a, b). The highly specific and sensitive fluorescence method of FALCK and HILLARP (see FALCK 1962; FALCK, HILLARP, THIEME and TORP 1962; CORRODI and HILLARP 1963, 1964) has now opened entirely new approaches in this field and it has become possible directly to demonstrate the presence and intraneuronal distribution of the adrenergic transmitter (cf. FALCK 1962; HAMBERGER and NORBERG 1963; HAMBERGER, NORBERG and SJÖQVIST 1963a, b; FALCK 1964; DAHLSTRÖM and FUXE 1964a; MALMFORS 1964a; NORBERG 1964). The sympathetic adrenergic neuron and the adrenergic innervation apparatus have now been extensively studied by means of this method and some fundamental characteristics of this neuron are discussed in the present paper.

[1] *Abbreviations used*: DA = dopamine; NA = noradrenaline; A = adrenaline; CA = catecholamine(s); 5-HT = 5-hydroxytryptamine; DOPA = 3,4-dihydroxyphenylalanine; MAO = monoamine oxidase.

II MATERIAL AND METHODS

Untreated cats (31) and rabbits (8) of both sexes and adult male albino rats (Sprague Dawley 80) were used for the experiments Other rats cats and rabbits were pretreated with drugs interfering with the CA metabolism in adrenergic nerves for details on treatment and numbers of animals see Table 1

The effects of denervation and decentralization were studied in the rat and cat The cervical sympathetic trunk was for this purpose extirpated below the superior cervical ganglion (1–20 days before killing the animal 17 rats 2 cats) or else this ganglion was extirpated together with the preganglionic trunk (2 days before killing the animals 6 rats 2 cats) In cats ([illegible]) experiments were also made where the postganglionic nerves from the inf mesenteric ganglia to the colon were cut

The following tissues were especially examined sympathetic ganglia (sup cervical stellate coeliac [rat rabbit and cat] inf mesenteric several lumbar ganglia [cat]) and spinal nodose submaxillary and ciliary ganglia intestine submaxillary and parotid glands heart urinary bladder vas deferens seminal vesicles prostatic glands pelvic plexus and iris

The cats and rats were anaesthetized the rats by ether and the cats by sodium pentobarbital (Nembutal Abbott 40 mg kg) and killed by bleeding out The rabbits were killed by an i v injection of air

Small pieces of the tissues were dissected out immediately after death and rapidly frozen in propene propane (4 1) cooled with liquid nitrogen They were dried *in vacuo* for 5–6 days treated with formaldehyde gas with optimum water content (HAMBERGER MALMFORS and SACHS 1964) at 80 C for 1 hour embedded in paraffin sectioned at 7–10 μ and mounted in Entellan (Merck) for fluorescence microscopy A few drops of xylene were added to the mounting medium for deparaffinization of the sections

The histochemical procedure used in this laboratory which mainly follows the procedure originally developed by FALCK has recently been described in detail by DAHLSTRÖM and FUXE (1964*a*) Rat iris

and pieces of rat mesentery were prepared also as stretch preparations mounted whole this technique is described in detail by MALMFORS (1964a) The iris preparations were studied in cooperation with Dr T MALMFORS and Dr CH SACHS A fluorescence microscope (Zeiss) with non fluorescent objectives was used with a Schott OG 4 or a Zeiss 50 filter (1 mm) The source of the activation light was an Osram HBO 200 high pressure mercury lamp with a 3 or 4 mm Schott BG 12 filter A dark field condenser for oil immersion was used for examination and photography The film used was Scopix G (Gevaert) and exposure times were generally between 15 and 60 sec

The wavelength giving maximum fluorescence activation was determined by means of a special fluorescence microscope built at this department by research engineer G Thieme The source of light was a high pressure xenon lamp (Osram XBO 1600) A water cooled 1 mm Schott BG 38 filter was used for heat absorption The light beam was focused on the entrance slit of a Zeiss M40 III monochromator On the exit side quartz lenses were arranged in such a way that the following microscope condensor (Zeiss light or dark field) projected an image of the entrance slit of the monochromator in the plane of the specimen In this way it was possible to illuminate a narrow strip of the field of view minimal slit widths could be used giving an illuminated breadth down to the diameter of a sympathetic ganglion cell A Wratten filter no 15 was used in the microscope tube as uv cut off

III THE OCCURRENCE AND INTRANEURONAL DISTRIBUTION OF THE ADRENERGIC TRANSMITTER

Normal tissues treated with formaldehyde gas according to the present method exhibited fluorescence of in principle three different types. Firstly, there were found different types of autofluorescence (cf. SJÖSTRAND 1944) that were unaffected by reserpine pretreatment and occurred also in tissues not treated with formaldehyde gas. Secondly, certain tissues developed on formaldehyde treatment a faint dirty green unspecific fluorescence, which also was unaffected by reserpine pretreatment. This background fluorescence, which could be due e.g. to reactions between tissue proteins and formaldehyde, was seldom seriously disturbing unless technical faults had been made. Thirdly, certain nerve cells and fibres showed a specific green to yellow green fluorescence which did not develop after reserpine pretreatment. There is little doubt that this type of fluorescence is due to the presence of NA (see chapter V). The specific green or yellow green fluorescence was found in the cell bodies of all or most of the ganglion cells in the sympathetic ganglia (Fig. 1–3) but has also been observed in nerve cells in the pelvic plexus (Fig. 28–29) (cf. FALCK, OWMAN and SJÖSTRAND pers. commun.) and the intramural ganglia of the bladder wall (HAMBERGER and NORBERG 1964). No fluorescence of this specific type was found in sensory (spinal, nodose), parasympathetic (ciliary, submaxillary [Fig. 13], gastrointestinal) or sympathetic cholinergic (HAMBERGER, NORBERG and SJÖQVIST 1963a, 1964, cf. SJÖQVIST 1962, 1963a, b) ganglion cells. The specific yellow fluorescence characteristic of 5-HT was never observed in nervous structures in the present material.

The fluorescent material in the ganglion cells appeared to be distributed diffusely in the cytoplasm. In the periphery of the cytoplasm there was sometimes found a rim of higher intensity. Sometimes such a zone was seen also around the nucleus. The nucleus never showed any fluorescence, unless diffusion had occurred due to technical error (Fig. 4).

The fluorescent ganglion cell populations were heterogeneous in

respect of their intensity (Figs 1–5) The majority of cell bodies exhibited a medium fluorescence while smaller proportions showed a higher or fainter intensity This heterogeneity was abolished neither by decentralization (rat and cat sup cervical ganglion 1 to 20 days) nor by treatment of the animals with the potent MAO inhibitor nialamide (Fig 3) The latter however gave in rat a slight general increase in fluorescence In sympathetic ganglia a few cells were found which were mostly located in clusters and much smaller than the ganglion cells They exhibited a specific green to yellow green fluorescence of higher intensity than the ganglion cell (NORBERG and UNGERSTEDT to be publ)

The dendrites and axons emanating from the cell bodies in the sympathetic ganglia showed a faint fluorescence of the specific type and could thus be followed for some distance from the cell body (Fig 7) When the fluorescence reaction was perfect bundles of such fluorescent axons were seen between the ganglion cells and could be traced to the nerve trunks emanating from the ganglia (Fig 5) Especially in the cat ganglia there were found also bundles of nerve fibres with faintly autofluorescent myelin sheaths but lacking specific fluorescence These fibres were probably preganglionic or belonged to the cholinergic sympathetic system The satellite cells were for the most part difficult to distinguish exhibiting only a very faint unspecific fluorescence

In the main peripheral nerve trunks the postganglionic adrenergic axons could usually not be observed or showed a very faint fluorescence They could be visualized by raising their NA content in some way this could be done by the administration of NA or its precursors DA or L DOPA after pretreatment with nialamide (HAMBERGER MALMFORS NORBERG and SACHS 1964 MALMFORS 1964*a*) (Figs 15–16) or by constriction or transection of the nerves which results in a piling up of the transmitter above the lesion (DAHLSTRÖM and FUXE 1964*b*)

In many tissues there could be found small nerves which when longitudinally cut exhibited smooth very faintly fluorescent fibres mixed to a greater or lesser degree with non fluorescent fibres In transverse sections the fibres could often be seen as small fluorescent dots among the non fluorescent fibres

In peripheral tissues known to have a sympathetic adrenergic innervation like smooth muscle in several locations (e g blood vessels [Figs 13 19–23] male internal genital organs [Figs 24–32] iris) and

glandular tissue (e g submaxillary [Figs 13 17] and parotid glands) there were found abundant nerve fibres of quite another type These ran in close proximity to the effector cells and exhibited a strong green to yellow green fluorescence They were fine and in appearance somewhat like a string of pearls with alternating thin segments—sometimes barely visible—and enlargements the varicosities which were brilliantly fluorescent (Figs 21 24–27) Varicose fibres of the same type representing a previously unknown system of synaptic terminals originating from adrenergic neurons (Fig 6 8–11) have been shown to exist in the sympathetic ganglia of the cat (HAMBERGER NORBERG and SJOQVIST 1963*b*) Such terminals have now been found also in the superior cervical ganglion of the rat and rabbit (NORBERG unpubl observations) the celiac ganglion of the rabbit (Fig 12) and around parasympathetic ganglion cells in both the intestinal wall (NORBERG 1964) and the wall of the urinary bladder (HAMBERGER and NORBERG 1964)

The fluorescent varicose terminals and the weakly fluorescent axons in the small intramural nerve bundles in the effector organs disappeared within 48 hours after the postganglionic nerve from the sup cervical ganglion (rat and cat) and the inf mesenteric ganglion (cat) had been cut In these cases the adrenergic axons proximal to the axotomy showed an increased fluorescence intensity Also all the cells finally achieved a fairly equal intensity No obvious changes on the other hand were observed in the cell bodies axons or terminals after decentralization (rat superior cervical ganglion 1 to 20 days) The variation in fluorescence intensity was also unaffected

The effects of drugs interfering with the monoamine metabolism have been studied with special reference to the reactions of the fluorescent cell bodies as compared to the terminals A detailed account of the pharmacological experiments will be given in a forthcoming paper Some observations of particular interest for the present work are reported in chapter V — There is convincing evidence that the cell bodies axons and terminals exhibiting the specific fluorescence described above belong to the adrenergic neurons (see chapter VI)

IV SOME CHARACTERISTICS OF THE ADRENERGIC TERMINAL

The fluorescent varicose nerve terminals could be easily studied in certain tissues, above all in vas deferens and in whole mounts of iris and mesentery. They have throughout their length and in all the tissues examined a characteristic appearance, being extremely fine and showing abundant varicosities but having no other special structures. All the pharmacological tests made so far—also by other investigators in this laboratory—have shown that the adrenergic terminal reacts uniformly throughout its entire length on depletion of the adrenergic transmitter by drugs (reserpine, m-tyrosine, α-methyl-m-tyrosine, aramine, guanethidine) acting by different mechanism. The fluorescence reappeared uniformly (although at quite different rates) also during the recovery stages after such depletion.

In whole mounts of iris and mesenterium it was possible to observe the actual transition from smooth axons with a very faint fluorescence into intensely fluorescent varicose fibres. Immediately before this transition the preterminal smooth axons in the nerve bundles often exhibited a somewhat increased intensity and a few varicosities could sometimes be seen particularly distinctly in the iris (MALMFORS and SACHS, 1964a, b) and the pelvic plexus. The fibres then left the nerves and passed into intimate contact with innervated cells, where they suddenly assumed the appearance of typical varicose terminals. The terminals at this point generally enter an autonomic ground plexus (HILLARP, 1946, 1959a, b; see also Chapter VI).

The varicosities were of varying length, showed great variations in thickness and were usually closely—but often more or less irregularly—spaced. Their thickness varied between 0.4 and 2 μ (generally 0.8 to 1.2 μ) and their length between 0.5 and 3 μ. They occurred in numbers of 15 to 30 per 100 μ fibre length. The thin segments between them varied in length but were mostly between 1 and 3 μ and down to about 0.2 μ thick. Approximately the same values were found for the air dried whole mounts of rats iris as for the freeze dried preparations of vas deferens (rat and guinea pig) and prostatic vesicle (rab

but). In the vessels and glands, however, the varicosities generally seem to be smaller and less pronounced. The terminals in the mouse iris, on the other hand, have unusually large varicosities (MALMFORS 1964a). It must be pointed out that all these figures are subject to considerable uncertainty, owing to the difficulty of performing the measurements.

The organization of the innervation apparatus varied somewhat in different tissues. The nerve terminals were generally arranged in a ground plexus in intimate contact with the effector cells (HILLARP 1946, 1959a). Each strand in the plexus generally contained several fibres, but in certain tissues most of the strands in the plexus contained only one varicose terminal. A typical ground plexus with for the most part several varicose fibres running in each strand was found in the walls of blood vessels (Fig. 21), iris, heart muscle—an exceptionally dense network has been found in the sinoauricular node (ANGELAKOS, FUXE and TORCHIANA 1963, Fig. 18)—the submaxillary (Fig. 15, 17) and parotid glands (NORBERG and OLSON, unpubl. observations), the cardiac sphincter of the stomach (NORBERG, unpubl. observations) and the trigone of the urinary bladder (Fig. 33; HAMBERGER and NORBERG 1964). Further microphotos are given in papers by FALCK (1962), DAHLSTRÖM and FUXE (1964 a, c), FUXE and SEDVALL (1964), HAMBERGER and NORBERG (1964) and MALMFORS (1964 a). This type of innervation has been found also in other tissues, such as the large hair arrector muscle in the cat (FUXE and NILSSON, pers. commun.) and the smooth muscle tissue of the nictitating membrane (MALMFORS 1964c) and the upper eyelid (MÜLLER muscle) in e.g. the rat (MALMFORS, pers. commun.).

The typical and dense ground plexus innervation of the dilator muscle of the rat iris (see figures in FALCK 1962, HAMBERGER, MALMFORS, NORBERG and SACHS 1964, MALMFORS 1964a, b) has been found to be directly superimposed on the outer surface of the single sheet of smooth muscle cells (MALMFORS 1964b). This is in full agreement with recent electron microscopical studies of the iris innervation performed in this laboratory (HÖKFELT and NILSSON, pers. commun.).

The nerves in the walls of blood vessels were found to be arranged in a special way. A typical ground plexus was found to be located on the border between the adventitia and the smooth muscle layer of the media (Fig. 13–16). Although this plexus showed great quantitative variation, it formed everywhere a finely or widely meshed

two dimensional network with for the most part several fine terminals running together in each strand This network surrounded the muscle layer throughout the entire length of the vessels In cross sections the plexus was seen to be directly superimposed on the outer surface of this layer not penetrating in between the muscle cells It is of great interest that the plexus was found to have this construction and localization also in large arteries with a thick media only in exceptional cases has it been observed to penetrate into this layer and then only quite superficially Mesenteric arteries and small intramural arteries in several tissues showed an extremely rich innervation with a very finely meshed ground plexus (Fig 19–20) The veins showed great variations with regard to innervation Sometimes veins were found with a ground plexus of high density surrounding the muscle layer while sometimes there were very few or no adrenergic fibres mostly however there was seen a scarce innervation in the form of a very widely meshed plexus (Fig 13–14) Other studies (FALCK 1962 FUXE and SEDVALL 1964) support this view of the general arrangement of the adrenergic vasomotor apparatus

In other places where smooth muscle is innervated by a typical ground plexus such as the nictitating membrane and the trigone of the urinary bladder it forms a three dimensional network with the meshes elongated in the direction of the muscle fibres This network penetrates the entire muscle layer and thus closely approaches the cells throughout the tissue In the adrenergically innervated glands the ground plexus forms a network that closely surrounds the acini (Fig 13 17) resembling in this way the vasomotor plexus Usually several terminals run together in each strand The terminal are often so fine and lie so closely together that it can be difficult to observe the individual fibres When the fluorescence reaction is perfect however they can be clearly observed at least at favourable places in the plexus

In contrast to most of the tissues so far considered the smooth muscle tissue in the vas deferens and seminal vesicle was found to have a ground plexus of another type Most of the plexus strands contain here only one varicose terminal (Fig 24–27 cf FALCK 1962 figures 12–14) The terminals run everywhere in the tissue between and along the muscle cells giving an innervation of high density It seems not impossible that most of the cells somewhere on their surface have contacts with or lie in close proximity to varicosities belonging to one or more terminals

Transitions between these two principal types of ground plexus were found e g in the pro tatic glands (Figs 28–32) and in such blood vessels as have sparse vasomotor terminals running in a widely meshed plexu Some of the strands in these showed two terminals and others a single terminal

V HISTOCHEMICAL AND PHARMACOLOGICAL CRITERIA FOR THE SPECIFICITY OF THE FLUORESCENCE REACTION

The chemical basis of the fluorescence reaction is by now fairly well known (FALCK, HILLARP, THIEME and TORP 1962, CORRODI and HILLARP 1963, 1964). The method is based on the principle that primary CA (such as DA and NA) and tryptamines (such as 5-HT) readily condense with formaldehyde to 1,2,3,4-tetrahydroisoquinolines and β-carbolines respectively, which are then in a protein-catalyzed reaction dehydrogenated to the corresponding intensely fluorescent 3,4-dihydro compounds. The essential point is that in order for the dehydrogenation step to take place readily the amines must be in very intimate contact with the protein, which is the case when they exist enclosed in the dried protein layer. This explains the ready conversion of the amines present in freeze- and air-dried tissues. The products that show an intense fluorescence in the solid state but not in aqueous solutions have activation peaks at about 410 mμ while the emission spectra of the isoquinolines and β-carbolines are different (peaks at 480 and 520–530 mμ respectively). This explains why fluorescence due to the presence of CA differs in colour from that due to 5-HT, the former being green or yellow-green and the latter yellow (with the equipment used). Another fact of interest in this connexion is that secondary amines such as A are converted to a significant extent to fluorescent products only if the formaldehyde treatment is prolonged (3 hours or more at +80 C). It is thus possible to differentiate between primary and secondary CA (cf. FALCK, HAGGENDAL and OWMAN 1963). Other biogenic compounds have been concluded either to occur in the tissues in too low concentrations to interfere with the demonstration of monoamines or not to give the specific type of fluorescence under the conditions used (FALCK 1962, FALCK, HILLARP, THIEME and TORP 1962, HILLARP, pers. commun.).

The method has been found to have a very high sensitivity and specificity and has been extensively used in studies of model systems and in studies on sections of both peripheral tissues and the central nervous system in mammals and other animals.

To evaluate the specificity of a fluorescence observed in a tissue it is essential, however, that certain histochemical and, whenever possible, pharmacological criteria be fulfilled. The specificity of the reaction and the use and significance of the specificity tests have been discussed in numerous papers (see e.g. CARLSSON, FALCK and HILLARP 1962; FALCK 1962; CORRODI and HILLARP 1963, 1964; CORRODI, HILLARP and JONSSON 1964). The data pertinent to these problems have recently been summarized in an investigation of the monoamines in the central nervous system (DAHLSTRÖM and FUXE 1964a). For this reason the experiments made in the present work are described below only briefly without any detailed discussion.

A. Histochemical criteria

1. *Conditions for the development of the fluorescence*

When the formaldehyde treatment was performed at + 80° C for 1 h the nerve cells and fibres described above—like models with a primary CA (DA and NA)—developed a specific green to yellow-green fluorescence. The intensity of the fluorescence was highly dependent on the amount of water present (HAMBERGER, MALMFORS and SACHS 1964). If too little water was present, no or only very faint fluorescence developed. With an increasing water content the intensity first increased to a maximal level but the structures then became blurred and indistinct owing to diffusion. The faint background fluorescence which may be due among other things to reactions between the formaldehyde and certain proteins and the autofluorescence occurring in certain nerve cells and other tissues were not influenced by the formaldehyde treatment; neither did they change appreciably when the water content was altered.

2. *Peak of activation and colour of the fluorescence*

The main peak of activation for the fluorescence developed in the nervous structures was the same as that of models with primary CA (i.e. c. 410 mμ, uncorrected) and the light emitted was always green to yellow-green (cf. CORRODI and HILLARP 1963, 1964). In these structures there was never found the pure yellow fluorescence characteristic of models with 5-HT and similar amines, and of mast cells, certain brain neurons and other cells containing 5-HT (FALCK 1962; BERTLER, FALCK and OWMAN 1963; DAHLSTRÖM and FUXE 1964a; OWMAN 1964; HILLARP pers. commun.). Under certain conditions

(high amine concentration long formaldehyde gas treatment and a formaldehyde gas saturated with water) further reactions can take place in model experiments resulting in a yellow or even orange fluorescence even for CA (HILLARP pers commun) This however has never been observed when the reaction is performed as described here The localization of the small amounts of A known to occur in sympathetic ganglia (KIPPEKAB CERVONI and FURCHGOTT 1962 MUSHOLL and VOGT 1958 HOLMSTEDT *et al* to be publ) will be discussed in a forthcoming paper It is quite clear however that the fluorescence observed in the ganglia treated for 1 h is due to a primary CA and not to a secondary CA like A

The spectra of fluorescent cell structures in tissue sections have been determined with the use of a microspectrophotometric technique developed by CASPERSSON It was found (RITZÉN pers commun) that the emission spectra of the structures showing specific fluorescence were practically identical with those of the compounds formed from DA and NA in models but distinctly different from that developed from 5 HT (CASPERSSON HILLARP and RITZEN to be publ)

3 *Sensitivity to irradiation with ultraviolet light*

The fluorescent products formed from CA and 5 HT are sensitive to u v light Irradiation of the nerve cells in the fluorescence microscope (Osram HBO 200 lamp 3 mm BG 12 filter) resulted within 10 minutes in a distinct decrease in the intensity of the fluorescence This decrease however was much slower than that characteristic of the 5 HT product in models (RITZÉN pers commun) and in the central 5 HT neurons (DAHLSTROM and FUXE 1964*a*) In these cases there is a very rapid initial decomposition followed by a slower phase

4 *Quenching of the fluorescence with water*

In aqueous solutions the fluorescent products from the reacting monoamines show at the most a weak fluorescence Even if the products—by being enclosed in or bound to the proteins—have become more or less insoluble water still considerably reduces their fluorescence (cf CORRODI HILLARP and JONSSON 1964) If the paraformaldehyde treatment had not been continued for more than 1 h a great deal of the fluorescent product in the nerve cells and terminals could be extracted by water but not by 40 % isopropanol in water It was found that the fluorescence developed in the cells decreased or disappeared when the sections were mounted in 40 % isopropanol in water and reappeared after dehydration and mounting in xylene

5 *Reduction to non fluorescent compounds*

A histochemical test of high specificity for CA and 5 HT is based on the finding (CORRODI HILLARP and JONSSON 1964) that the fluorescent compounds are readily reduced by sodium borohydride under mild conditions to the corresponding 1 2 3 4 tetrahydro compounds These are non fluorescent but can be converted again to the fluorescent 3 4 dihydro products on renewed formaldehyde treatment No obvious changes in non specific fluorescence have been found to occur as a result of this treatment This test was applied to nerve cells from sympathetic ganglia treated with formaldehyde gas at +80 C for 1 to 3 h It was found that 0 03 % sodium borohydride in 95 % isopropanol caused the green to yellow green fluorescence to disappear within 2 minutes although no changes were observed in the non specific fluorescence Treatment with the solvent alone did not appreciably influence the fluorescence The fluorescence could be regenerated by renewed formaldehyde treatment for 1 h at +80 C

These experiments were made in cooperation with Dr G JONSSON of this department

B Pharmacological criteria

The desirability of using pharmacological tests to evaluate the specificity of the fluorescence in all tissues studied has been repeatedly emphasized (CARLSSON FALCK and HILLARP 1962 FALCK 1962 CARLSSON FALCK FUXE and HILLARP 1964) Such experiments were therefore performed on peripheral nerve cells and terminals in the tissues examined (Table 1) A detailed account of the effect of these and several other drugs will be published separately

1 *Depletion and repletion of the NA store of nerve cells and terminals*

A large dose of reserpine (5 mg/kg) which is known to deplete the CA stores in the nervous system (cf CARLSSON 1964*b*) resulted in an almost complete absence of specific fluorescence in the nerve cells and terminals as early as 3 h after administration of the drug An incipient reappearance of the fluorescence was observed in the nerve cells after 12 to 24 h while the terminals began to recover much more slowly (48 to 96 h) The effect of this drug on the cell bodies as compared with the terminals was studied in detail in the rat The strong fluorescence of the terminals had to a large extent or practically completely disappeared 2 to 4 h after the administration of doses of

0 1 to 0 25 mg/kg (No fluorescence was observed in the non terminal axons and terminals 4 h after a dose of 0 5 mg/kg) The cell bodies reacted about as rapidly as the terminals but required about ten times higher doses for considerable to complete disappearance of their specific fluorescence

The reappearance of fluorescence in cell bodies and terminals after total depletion by reserpine (5 mg/kg) was studied in the rat The first reappearance in the cell bodies occurred at 12 h and a more or less complete recovery was found 48 h after administration of the drug The terminals on the other hand showed at most a very weak fluorescence at 48 to 72 h and recovery to roughly normal fluorescence intensity was not observed until several days later

The administration of other drugs (*m* tyrosine α methyl *m* tyrosine aramine) known to deplete the stores of NA in adrenergic nerves (cf Carlsson Falck and Hillarp 1962 Falck 1962 Andén and Magnusson 1964 Carlsson 1964*a*) also resulted in the disappearance of—or a very marked decrease in—the fluorescence of the entire neuron Recovery of the fluorescence after α methyl *m* tyrosine (rat) began much more rapidly in the cell bodies (about 12 h) than in the terminals (after 24 h)

Potent MAO inhibitors have been reported to give an increase in the NA content of the adrenergic nerves in rat tissues but a decrease or no certain changes in the cat (Goldberg and Shideman 1962 Sanan and Vogt 1962 Davey Farmer and Reinert 1963) When a single dose (100 to 500 mg/kg of nialamide) was given to rats a slight but definite increase in the fluorescence of the cell bodies the non terminal axons and terminals was observed within 6 h No distinct change in any part of the adrenergic neuron could be seen however in the cat

2 *Normal occurrence and drug induced increase of CA in various types of peripheral nerve cells and fibres*

In the sympathetic ganglia specific fluorescence occurs in those nerve cells that from their number and location may be assumed to belong to adrenergic neurons while the sympathetic cholinergic parasympathetic and sensory neurons are quite devoid of such fluorescence (see also Hamberger Norberg and Sjoqvist 1963*a* 1964) This in itself is good support for the specificity of the fluorescence reaction The marked difference between the cell types is emphasized still further

TABLE 1

Drug	Species	Dose mg/kg body weight	Time before killing the animals (hours)
Number of animals within brackets			
Reserpine	rat (10)	2.5–5 i.p.	8–24
	rabbit (3)	2 i.v.	12–16
	rat (110)	0.1–10 i.p.	1–[illegible]
Nialamide	rat (13)	100–300 i.p.	4–7
	rat (21)	100–[illegible]00 i.p.	4–[illegible]
α-Methyl-m-tyrosine	rat (28)	2 × 400 i.p. at intervals of [illegible] h	1–[illegible] (after the second dose)
m-Tyrosine	rat ([illegible])	2 × 400 i.p. at intervals of 2 h	[illegible] (after the last dose)
Aramine (metahydroxynorephedrine)	cat (2)	2 × [illegible] i.p. at intervals of 3 h	3 (after the second dose)
	rat (10)	2 i.p.	[illegible]
L-DOPA	rat (10)	[illegible] i.p.	2–4
Nialamide	rat (6)	[illegible]00 i.p.	[illegible]
L-DOPA		[illegible] i.p.	[illegible]
Nialamide	rat ([illegible])	[illegible] i.p.	[illegible]
DA		[illegible] i.p.	1–2
Nialamide	rat ([illegible])	100 i.p.	[illegible]
NA		0.1 i.v.	1
α-Methyl-DOPA	rat ([illegible])	400 i.p.	2–[illegible]
α-Methyl-NA	rat ([illegible])	0.1 i.v.	[illegible]

by other observations. It has thus been found that not only the adrenergic nerves but the entire adrenergic neuron has a specific mechanism localized to the level of the cell membrane for the uptake and concentration of NA (HAMBERGER, MALMFORS, NORBERG and SACHS 1964). Due to this mechanism, which is insensitive to reserpine but blocked by cocaine and certain other drugs (HILLARP and MALMFORS 1964; MALMFORS 1965), amines accumulate rapidly to very high levels in the preterminal axons and terminals after administration of NA and DA, especially when administered to animals pretreated with MAO inhibitors. Such accumulation can also

be obtained by administration of the precursor L-DOPA (20 mg/kg i p) The entire non terminal adrenergic axon which normally exhibits no or at most a weak fluorescence can thus be easily demonstrated and distinguished from the other types of nerve fibres which lack this mechanism The administration of NA DA or L-DOPA after pretreatment with a MAO inhibitor (or of α methyl NA without such pretreatment) has also been found to cause a marked increase in the intensity of the normally fluorescent nerve cells present in the sympathetic ganglia while the cholinergic and sensory nerve cells show no specific fluorescence Adrenergic nerve cells can in this way be distinguished from other peripheral ganglion cells

The results of the histochemical and pharmacological experiments permit the definite conclusion that the green or yellow-green fluorescence of nerve cells and terminals following formaldehyde treatment is due to the presence of a primary CA

It is clearly very probable that the different parts of the adrenergic neurons cell bodies smooth axons and varicose terminals contain the same primary CA NA This view is supported by the results of earlier biochemical studies which indicate that NA is present not only in the adrenergic terminals but in the entire adrenergic neuron (VON EULER 1956) Other biochemical data give further support to this view NA is thus the only primary CA found in significant amounts in the sympathetic ganglia (HOLMSTEDT *et al* to be publ) A recent analysis has furthermore shown that very little DA is present in the superior cervical ganglion of the rabbit (0.2 μg/g against 7 2 μg/g of NA HÄGGENDAL pers commun) Fairly high concentrations of DA have been found in bovine splenic nerves (SCHÜMANN 1956 EULER and LISHAJKO 1957) There is little doubt however as suggested by VON EULER and LISHAJKO (1958) and demonstrated by BERTLER FALCK HILLARP ROSENGREN and TORP (1959) that this DA is localized in chromaffin cells which belong to a special system of monoamine forming mast cells (ADAMS RAY DAHLSTROM FUXE and HILLARP 1964) Furthermore there is good evidence for the view that the first step in the monoamine synthesis is rate limiting (see BERTLER HILLARP and ROSENGREN 1960 AXELROD 1963 CARLSSON CORRODI and WALDECK 1963 UDENFRIEND and ZALTZMAN NIRENBERG 1963 CARLSSON and CORRODI 1964 NAGATSU LEWITT and UDENFRIEND 1964) The precursors L DOPA and DA both of which give the fluorescence reaction can

therefore not be normally expected to accumulate in the adrenergic neuron This has been confirmed for the closely related adrenal medullary cell (BERTLER HILLARP and ROSENGREN 1960 HALL HILLARP and THIEME 1961)

The above mentioned results and arguments leave little doubt that NA is the only primary CA present in the adrenergic neuron in significant amounts from the histochemical point of view and responsible for the specific fluorescence seen with the present method

VI GENERAL DISCUSSION

With the histochemical fluorescence method used it has for the first time been possible directly to demonstrate the localization of the stores of the adrenergic transmitter NA in the tissues namely in the thin nerve terminals above all in their varicosities (FALCK 1962) The present results clearly show that not only the varicose nerve terminals but also fluorescent ganglion cell bodies and non terminal axons contain a primary CA in all probability NA (see chapter V) Strong support has already been presented for the view that the fluorescent sympathetic ganglion cells are adrenergic (HAMBERGER NORBERG and SJOQVIST 1963a 1964) —All these nervous structures can be assumed to belong to the same neurons Several observations support this view Confirming the observations made by FALCK (1962) no smooth non terminal axons or varicose terminals with specific fluorescence could be observed in tissues where the postganglionic sympathetic nerves had been selectively cut 48 hours previously It was moreover possible directly to observe the transition from non terminal axons with faint fluorescence into the intensely fluorescent adrenergic terminals Taken together these data clearly show that the nervous structures exhibiting specific fluorescence belong to the adrenergic neurons

There is known from biochemical studies to exist somewhere in the adrenergic nerves of the tissues a specific mechanism for the uptake of NA (MUSCHOLL 1960 HERTTING AXELROD KOPIN and WHITBY 1961 AXELROD HERTTING and POTTER 1962) This mechanism has been shown to be present not only in the terminals but also in the entire postganglionic axon and in the cell body of the adrenergic neuron (HAMBERGER MALMFORS NORBERG and SACHS 1964 MALMFORS 1964a) The mechanism has furthermore been *shown to have the properties of a pump localized to the level of the* cell membrane and it can concentrate NA and similar CA very efficiently and to high intraneuronal levels [1]

[1] Thanks to these findings we have now simple procedures available to bring about a specific accumulation of CA in the entire adrenergic neuron Since the amines accumulated can easily be demonstrated and since other types of peripheral nerve cells and fibres have no such mechanism for uptake and concentration it is possible readily and specifically to demonstrate both the adrenergic axons and the adrenergic nerve cells throughout the body

The sensitivity of the fluorescence method is very high. Experiments with model systems in this laboratory indicate that NA present in a 10 μ thick protein layer can be detected in concentrations of about 10–50 μg/g (HILLARP 1964, pers. commun.). This agrees well with the findings in the present paper. The cytoplasm of the nerve cells in 10 μ thick sections of the superior cervical ganglion exhibits a fluorescence which varies considerably in intensity from very faint to fairly high. This ganglion in rabbit and cat has a NA content of 7–8 μg/g (KIRPEKAR, CERVONI and FURCHGOTT 1962; PEINERT 1963; HAGGENDAL, pers. commun.). Assuming that the perinuclear cytoplasm of the adrenergic cells represents about one third to one quarter of the weight of the ganglion, the average concentration in this part of the neuron should be about 20–30 μg/g wet weight (some of the NA, however, is present in the adrenergic synaptic terminals, dendrites and postganglionic axons). It thus seems probable that the ganglion cells with the lowest and highest fluorescence intensity contain NA in the order of about 10 and 100 μg/g (0.001 and 0.01 %) respectively. This can be compared with the figure 0.5 % for the average concentration of ribonucleic acid in a spinal ganglion cell (cf. HYDÉN 1960). Since there are approximately 120 000 nerve cells (BILLINGSLEY and RANSON 1918–1919) in the superior cervical ganglion of the cat (wet weight about 12 mg, HOLMSTEDT *et al.*, to be publ.), the amine content is on the average somewhat less than 0.7×10^{-4} μg/cell. Less than one picogram distributed throughout the entire perinuclear cytoplasm (volume about 10 000 to 20 000 μ^3) of a nerve cell can thus be detected with the fluorescence method used. It may be concluded that the method has an exceptionally high sensitivity and compares favourably with many other histochemical and cytochemical methods.

The postganglionic non-terminal axons can usually not be demonstrated in peripheral mixed somatic and autonomic nerves unless their amine content is experimentally raised (see DAHLSTRÖM and FUXE 1964b; HAMBERGER, MALMFORS, NORBERG and SACHS 1964). Calculating from the biochemical data available (FULEP 1956; MUSCHOLL and VOGT 1958), the NA concentration in this part of the adrenergic neuron would seem to be in the same order of magnitude as that of the cell bodies. The present studies show that the axons running in the small nerve bundles in the innervated tissues also have a low transmitter concentration; this, however, is often high enough to permit detection and seems gradually to increase in the pre-terminal

part Taking into account the thin dimensions of the axons and the background fluorescence concentrations of 100–500 $\mu g/g$ wet weight would seem to be probable

The terminal parts of the axons are of quite different appearance with abundant elongated varicosities showing an intense fluorescence These are highly characteristic of those parts of the axons which are in close contact with the effector cells although here and there a few such varicosities may occur even in the preterminal axons It has sometimes been suggested that the varicosities are artifacts (e g RICHARDSON 1958) This possibility however can be ruled out as they can be clearly visualized with various quite different methods of preparation (HILLARP 1946 1959a b FALCK 1962) Especially on whole mounts of thin tissues such as mouse and rat iris (MALMFORS 1964a) the appearance of long stretches of the terminals can be studied clearly and in detail It can be concluded that the concentration of the transmitter in the terminals—especially in their varicosities—is very high since they exhibit in spite of their small dimensions an intense fluorescence There is little doubt that the overwhelming proportion of the tissue store of NA which was shown in the classical studies of EULER and coworkers (cf EULER 1956 1961) to be located somewhere in the terminal part of the adrenergic fibres is in fact localized in the varicosities Both the direct fluorescence microscopical observations and data on the NA content of various tissues (see e g EULER 1956 KIRPEKAR CERVONI and FURCHGOTT 1962 SJOSTRAND 1962 ANDÉN MAGNUSSON and WALDECK 1964 HAGGENDAL and LINDQVIST 1964) in relation to the size and distribution of the varicosities permit the definite conclusion that the concentration of the transmitter in these structures is of a quite different order of magnitude from that in other parts of the neuron Although such calculations are extremely approximative a value of 10 000 $\mu g/g$ would seem to be in the right order For several reasons (e g quenching of the fluorescence and possible low yields of the fluorescent 3 4 dihydro isoquinoline due to the high amine concentrations see CORRODI and HILLARP 1963 1964) this value may be rather too low than too high

There is good evidence that the amine storage granules which have been extensively studied by EULER and co workers (cf STJARNE 1964) are formed in the cell bodies of the monoaminergic neurons and then transported down to the terminals (DAHLSTROM and FUXE 1964 b d DAHLSTROM FUXE and HILLARP 1964 DAHLSTROM 1964) There is

furthermore good histochemical evidence that most of the amines are stored in these granules and thus protected from destruction by MAO (HAMBERGER, MALMFORS, NORBERG and SACHS 1964; HILLARP and MALMFORS 1964; MALMFORS 1964a). This explains the intraneuronal distribution of NA with low concentrations in the cell bodies and axons down to their preterminal part—but within this region often a somewhat higher concentration—and a very high accumulation in the terminals. Other results agree with this picture. Results have thus been obtained in this laboratory (MALMFORS 1963; FUXE pers. commun.) which indicate that monoaminergic neurons with short axons can have markedly higher amine concentrations in their non-terminal axons and even in their cell bodies than those with long axons. This suggests a piling up of the amine granules, such as is found after the constriction of adrenergic axons (DAHLSTRÖM and FUXE 1964b). The heterogeneity of the ganglion cells with respect to NA content may thus have an explanation. An extensive terminal system could require a high production and down transport of transmitter granules, which might leave a lower content in the cell bodies. Studies on the accumulation rate of the granules above a constriction of adrenergic axons clearly suggest that different neurons have markedly different rates of granule formation (DAHLSTRÖM 1964).

ERÄNKÖ and HÄRKÖNEN (1963) have described granular structures in ganglion cells, taking this as support for the view that the CA in the cells exists partly in granules and partly in soluble form. There is strong evidence, however, against this view. The occurrence of such granular structures is, according to our observations on an extensive material of sympathetic ganglia, the exception rather than the rule, and when the technical procedure has been perfect, we have found no cytological indications whatever to suggest that NA is localized in granules observable in the fluorescence microscope. It may be suggested that the granular appearance is caused partly by varicosities belonging to adrenergic synaptic terminals lying on the surface of the cell bodies and partly by artifacts.

There is strong indication, however, that the adrenergic transmitter normally does not occur "free" in the cytoplasm but must be stored in the amine granules, at least to the very largest extent (HAMBERGER, MALMFORS, NORBERG and SACHS 1964; MALMFORS 1964a). Obviously the transmitter granules themselves are far too small to be detected with the fluorescence method. On the other hand studies both on models (CORRODI and HILLARP 1963, 1964) and on

the uptake and accumulation of CA in adrenergic neurons clearly show that the fluorescence method demonstrates not only CA that is bound to a lipoprotein carrier in the granules—as recently claimed by CSILLIK (1964)—but also for instance the NA that under certain experimental conditions is taken up and accumulated in the cytoplasm outside the granules

Adrenergic and cholinergic neurons have certain fundamental characteristics and properties in common It is well known that both form and store their respective transmitters above all in the terminal parts of the axons The cholinergic fibres show a high acetylcholin esterase activity which together with choline acetylase seems to be involved in the regulation of acetylcholine synthesis and content (cf KOELLE and KOELLE 1959 MACINTOSH 1959 1963 SJOQVIST 1962) It has been shown histochemically that the adrenergic ganglion cells contain MAO (KOELLE and VALK 1954 NORBERG unpubl observations) and there is a great deal of indirect evidence that the enzyme is present throughout the entire adrenergic terminal (cf CARLSSON 1964*b* HAMBERGER MALMFORS NORBERG and SACHS 1964 HILLARP and MALMFORS 1964 MALMFORS 1964*a*) This enzyme seems to be involved in the amine metabolism of monoaminergic neurons (cf CARLSSON 1964*a*) The entire adrenergic neuron has the basic property of taking up and concentrating NA by a mechanism localized to the level of the cell membrane (see above) This mechanism is highly efficient not only in the terminals but also in the non terminal axons and in the cell bodies The cholinergic neurons apparently have no corresponding mechanism i e no capacity to reabsorb released acetylcholine They have instead an efficient mechanism for the uptake of choline which is necessary for the formation of the transmitter (cf MACINTOSH 1959 1963)

The classical observations of CANNON and ROSENBLUETH (1937 see also ROSENBLUETH 1950) concerning spatial and temporal summation in the autonomic neuro effector systems were given a logical explanation when a new concept was presented of the construction and functional organization of the autonomic neuro effector systems (HILLARP 1946) It was found that terminals from different neurons converge to and innervate one and the same effector cell group A great deal of evidence has accumulated in favour of this concept during the past 20 years (HILLARP 1959*a* HERZOG 1960 1963 FALCK 1962) According to this view the post ganglionic axons finally enter

the so called autonomic ground plexus and are transformed into thin terminals with abundant varicosities This plexus is a network with a mesh of varying fineness built of anastomosing strands of Schwann cells with usually two or more terminals running for considerable distances from strand to strand The absence of special nerve endings the characteristic appearance of the terminals the intimate connexions between the effector cells and the plexus and above all certain observations which strongly supported the view that the plexus is a closed terminal formation justified the conclusion that the ground plexus is the actual innervation structure and that the varicose axon ramifications are true terminals which release the transmitter along their entire lengths and in close proximity to the effector cells This explained the peculiar construction of the plexus —particularly the existence of more than one terminal in each strand —and the observations on convergence mentioned above

Important parts of this theory have been confirmed by electron microscopy There is thus no longer any doubt that the innervation apparatus is formed by terminal axons which closely approach the effector cells and that usually two or more such axons run together in the same Schwann cell (CAESAR EDWARDS and RUSKA 1957 FAWCETT and SELBY 1958 BENCOSME 1959 RICHARDSON 1960 GANSLER 1961 RUSKA and RUSKA 1961 ZELANDER EKHOLM and EDLUND 1962 THAEMERT 1963 APPENZELLER 1964) Of special significance is the fact that no contradictory observations have been made in studies on the adrenergic innervation of the iris dilator muscle by the present fluorescence method and electron microscopy (NILSSON 1964 HOKFELT and NILSSON pers commun) A comparison of the observations made in studies on the arterial innervation by the fluorescence method in this laboratory and by electron microscopy in other laboratories conclusively proves that both methods demonstrate the same innervation structures (see below) There is also little doubt that the terminal axons in the vas deferens described by writers using electron microscopy (e g MERRILLEES BURNSTOCK and HOLMAN 1963) are identical with the terminals that can be readily visualized with the use of the fluorescence method It is of interest furthermore that MERRILLEES BURNSTOCK and HOLMAN (1963) on the basis of their neurophysiological and electron microscopical observations have now adopted the view that the transmitter is released along the terminal axons The fundamental principle of convergence has received strong support from neurophysiological studies (see HILLARP

1959a BURNSTOCK and HOLMAN 1961 BURNSTOCK HOLMAN and PROSSER 1963 KURIYAMA 1963)

Electron micrographs have sometimes been interpreted as showing that the innervation is provided by individual nerve endings RICHARDSON (1962) concluded from electron micro copical investigations of rat vas deferens that there exist in this tissue numerous true ending —in both the anatomical and the physiological sen e—and that every smooth muscle cell has an ending A comparison between the actual observations of RICHARDSON and those made in the pre ent work hows however that the true nerve endings could be in fact identical with the varicosities of the adrenergic terminals In certain places (e g iris and some blood vessels) the plexus forms so dense a networl in relation to the number of effector cells that it is quite out of proportion for an innervation by individual endings The entire construction of these impressive plexu es of terminals is strong evidence for the view that such endings cannot play any important role It has also been pointed out (ZELANDER EKHOLM and EDLUND 1962) that since no serial sections have been studied it is impo sible to draw the conclusion that the structures seen by electron microscopy are true nerve endings and not varicosities belonging to nerve terminals It is much more probable that the structures assumed to be nerve endings are mostly varicosities in close contact with the effector cells and perhaps forming junctions similar to synapses (see e g figures in THAEMERT 1963)

Electron microscopical studies on the innervation of various arteries have al o failed to reveal any nerve endings within the muscle layer and have clearly shown that small bundles of terminal axons are present only on the outer surface of this layer the muscle layer it self being devoid of innervation (PEASE and MOLINARI 1960 LEVER and ESTERHUIZEN 1961 SAMARASINGHE 1962 APPENZELLER 1964) Due to the peculiar topography of the ground plexus it is possible to make a direct comparison between the results obtained by the present method and by electron microscopy proving conclusively that the two methods demonstrate identical structures of innervation This follows also from the compari ons between light and electron micros copy (BENCOSME 1959 HOKFELT and NILSSON pers commun) With the present histochemical method no certain nerve endings in the above mentioned sense could be observed not even when the innervation was very rich and easy to examine It is obvious however that the terminals must end somewhere Such endings were probably

seen by FALCK (1962) They have been clearly demonstrated in a study in the iris on the terminals belonging to single post ganglionic sympathetic neurons after subtotal denervation (MALMFORS and SACHS 1964b) Compared to the number of smooth muscle cells they are very few This speaks against the view that they play an important role in the innervation of the cells Such studies on whole mounts of rat iris have shown that the terminals are long branch frequently and have the same appearance throughout their length and that the terminals which run together in the strands of the ground plexus must belong to different neurons This is important verification of the principle of convergence

Since it is now possible directly to visualize the intraneuronal distribution of the adrenergic transmitter—and in particular the NA stores in the nerve terminals—the autonomic innervation apparatus can be studied in an entirely new way The observations made in this and other studies from this laboratory and by FALCK (1962) all strongly support HILLARP's theory (1946 1959a) Some very important parts of this theory have now been directly verified thanks e g to the fact that the present method has made it possible to study the entire extension of the innervation apparatus in thick sections or in preparations mounted as a whole The general construction of the ground plexus and its relation to the effector cells the transformation of the axons into characteristic terminals two or more of which usually run together for long distances in the strands of the plexus and above all the basic assumption that the ground plexus is a closed terminal formation have been verified The construction of the ground plexus can be studied especially well in blood vessels where it forms a very finely or more widely meshed network containing one two or more terminals in each strand The plexus here forms a two dimensional network which surrounds and is directly *superimposed on the outer surface of the muscle layer and can be* followed for the entire length of e g small arteries This directly shows that the plexus has the character of a closed terminal formation

The extensive studies carried out on the peripheral autonomic nervous system both with neurohistological methods (see HILLARP 1959a) and with the present fluorescence method have shown that all autonomic nerve fibres are transformed in their terminal part in an identical and very characteristic way they become very thin and abundant varicosities appear along their entire length The occurrence of this characteristic transformation just at the point where the

terminal axon ramifications closely approach the post synaptic neurons or the effector cells strongly supports the view that these ramifications are true terminals. This has been discussed at length by HILLARP (1946, 1959a). It seems now clear that the adrenergic—and probably also the cholinergic—terminals not only have a typical morphology and topography in relation to the effector cells but also have another characteristic of the greatest significance, namely a very high accumulation of the transmitter in the varicosities throughout the lengths of the terminals. This has been found to be the general rule for the sympathetic adrenergic terminals in the autonomic ganglia and peripheral tissues as well as for terminals belonging to monoamine containing neurons in the central and peripheral nervous system of vertebrates and invertebrates (cf. e.g. CARLSSON, FALCK and HILLARP 1962, DAHL, FALCK, LINDQVIST and v. MECKLENBURG 1962, DAHL, FALCK, v. MECKLENBURG and MYHRBERG 1963, HAMBERGER, NORBERG and SJÖQVIST 1963b, MALMFORS 1963, CARLSSON, FALCK, FUXE and HILLARP 1964, DAHLSTRÖM and FUXE 1964e, FUXE 1964, HAMBERGER and NORBERG 1964, NORBERG 1964). Furthermore, the adrenergic terminal has in all cases examined been found to react uniformly throughout its entire length to drugs and axotomy (see also MALMFORS 1964a, MALMFORS and SACHS 1964a) and no other special structures than the varicosities have been observed. They have also a highly efficient mechanism at the level of the axon membrane for the uptake and concentration of administered NA or the reabsorption of released transmitter (see above). Taken together, these findings represent very strong evidence that the varicose terminal parts of the axons are true terminals which release the transmitter stored in them. It seems highly probable that this release takes place chiefly or even exclusively from the varicosities, where most of the transmitter is accumulated. Direct evidence for this view has been provided by studies (MALMFORS 1964d) on the disappearance of NA from the terminals in iris on electrical stimulation of the cervical sympathetic trunk after treatment with certain recently developed inhibitors of the amine synthesis (CARLSSON, CORRODI and WALDECK 1963, CARLSSON and CORRODI 1964). Electron microscopical examinations of serial sections through the superior cervical ganglion (ELFVIN 1963) have directly demonstrated that the preganglionic terminals make synaptic contacts with the postganglionic neurons only via the varicosities. This is obviously one of the most important pieces of evidence we have for the role of the varicosities in transmitter release.

It has also been shown by electron microscopy that varicosities—called boutons en passage—exist along terminals in the brain and constitute the presynaptic structures of these terminals (WESTRUM and BLACKSTAD 1962, BLACKSTAD 1963). There is furthermore strong evidence that the varicosities—in contrast to the thin axon segments between them—not only have a very high accumulation of the transmitter but contain large numbers of "synaptic vesicles" (HOKFELT and NILSSON, pers. commun.). They also seem to be partially unensheathed by the Schwann cells, a construction which suggests transmitter release. There is thus little doubt that the varicosities are specialized structures for storage and release of the transmitter.

VII SUMMARY

The adrenergic transmitter can now be visualized in tissue sections by means of a sensitive and highly specific fluorescence method which in this investigation has been applied to a study of the sympathetic adrenergic neuron in different species. The specificity of the method was established by a combination of histochemical and pharmacological criteria and control tests.

The investigation provides evidence that not only the nerve terminals but the entire sympathetic adrenergic neuron contains a primary CA. All evidence supports the conclusion that—of the monoamines that can be demonstrated with the method used—only NA normally exists in the adrenergic neuron in histochemically significant amounts. No support was obtained for the view that the NA released on stimulation of the adrenergic nerves is derived from any storage site outside the adrenergic nerves. Adrenergic ganglion cells differ from other types of nerve cells by their ability to take up NA and some of its precursors. This specific mechanism for the intracellular accumulation of NA does not exist in other types of neurons. A considerable variation in amine content has been found among the cells and the cause of this heterogeneity is discussed. The concentration of the transmitter in the cell bodies seems to be in the order of 10–100 µg/g. Adrenergic ganglion cells have been found not only in the classical sympathetic ganglia but also in such other places as the intramural ganglia of the bladder wall and—confirming the observations of FALCK, OWMAN and SJÖSTRAND—the pelvic plexus.

In the non-terminal parts of the axons the amine concentrations seem to be in the same order of magnitude as in the ganglion cell, with a slight increase in the preterminal region in the neighbourhood of the effector cells. The thin adrenergic terminals, whose abundant varicosities give them a typical appearance, are in close contact with the effector cells. They have an extremely high concentration of NA. A value of about 10 000 µg/g seems to be in the right order of magnitude for the varicosities. Fibres of this kind have been found in such effector tissues as blood vessels, iris and certain glands and genital

organs The same type of terminal has in addition been previously found to represent a system of adrenergic synaptic terminals occurring in sympathetic ganglia and around parasympathetic ganglion cells in the intestine and the wall of the urinary bladder

The axons assume the typical appearance of varicose terminals precisely where they establish contact with the effector cells and enter the autonomic ground plexus The latter was found to have the construction described by HILLARP (1946 1959a) the plexus consists of a network of Schwann cells which contain two or more varicose terminals running from strand to strand True nerve endings have been observed only exceptionally and are not considered to play any role in transmitter release The very numerous nerve endings reported in electron microscopical investigations are interpreted as being the varicosities of autonomic terminals Strong evidence is adduced that the varicosities of both the adrenergic and cholinergic nerve terminals are specialized structures for the storage and release of the transmitter

General conclusions

1 Neuronal NA outside the central nervous system is localized in all parts of the classical sympathetic adrenergic neuron

2 The existence of peripherally located adrenergic neurons and especially the finding of a system of adrenergic synaptic terminals in the autonomic ganglia indicate that the anatomical and functional organization of the autonomic nervous system is more complex than hitherto realized

3 It is concluded that the adrenergic transmitter is released along specialized nerve terminals from varicosities which are the main storage sites of the intraneuronal NA

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organs The same type of terminal has in addition been previously found to represent a system of adrenergic synaptic terminals occurring in sympathetic ganglia and around parasympathetic ganglion cells in the intestine and the wall of the urinary bladder

The axons assume the typical appearance of varicose terminals precisely where they establish contact with the effector cells and enter the autonomic ground plexus The latter was found to have the construction described by HILLARP (1946 1959a) the plexus consists of a network of Schwann cells which contain two or more varicose terminals running from strand to strand True nerve endings have been observed only exceptionally and are not considered to play any role in transmitter release The very numerous nerve endings reported in electron microscopical investigations are interpreted as being the varicosities of autonomic terminals Strong evidence is adduced that the varicosities of both the adrenergic and cholinergic nerve terminals are specialized structures for the storage and release of the transmitter

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FIGURES

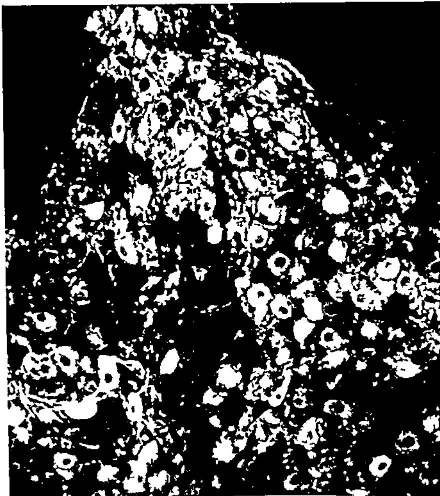


FIG 1 "th lumt ar gangl cat untreated Nerve cell bo lies of highly varying fluore cence inten ity are seen a f w be ng completely devoid of specific fluores cence Between the n rve c lls are seen fluorescent nerve processes × 180

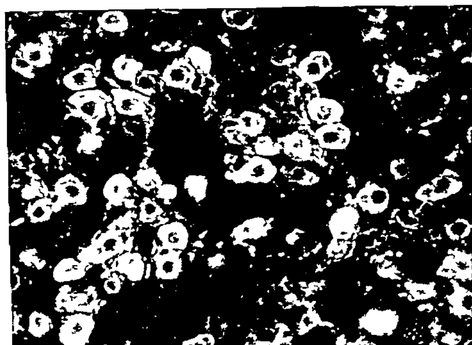


Fig – Sup cerv gangl rat untreated The nerve cell bodies exhibit fluorescence of varying intensity while the nuclei are essentially non fluorescent Intensely fluorescent adrenergic terminals are seen in the wall of a small artery ( ) – 80

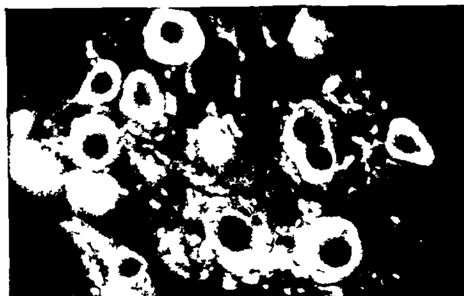


Fig 3 Sup cerv gangl rat nialamid 00 mg kg i p 5 h The fluorescence of the cell bodies is of varying intensity in spite of treatment with a potent MAO inhibitor No granules can be observed The slightly irregular distribution of the fluorescent material is due to a slight freeze artifact Note the non fluorescent nuclei and the binucleated cell 80

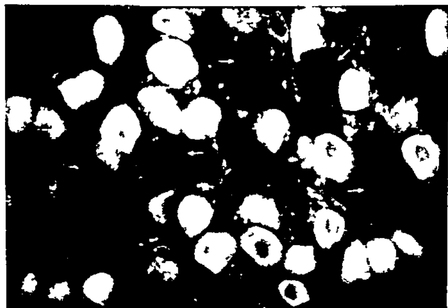


FIG 4 "tl lumbar gangl cat untr ated \ lrenergic cell bod s of varying fluorescence inten its and presumably cholinergic non fluoresc nt ( ) cell bodies are seen Small autofluorescent granules can be obs rved in the latter Due to a sl gt t liffusion (artifact) tl nucl i and especially the nu leol show some degree of fluorescence × 3 0

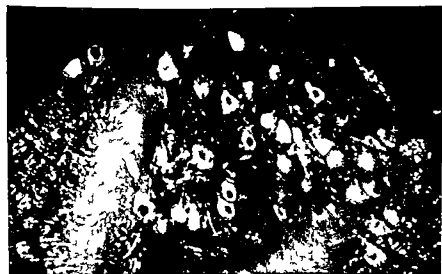


FIG 5 tl lumbar gangl cat untreated In ad lition to adrenergic and pre umably holine gic nerve cell bod es are n bundles of fluorescent (adren rgic A) an l non fluorescent (cholinergic? B) n n terminal axons × 180

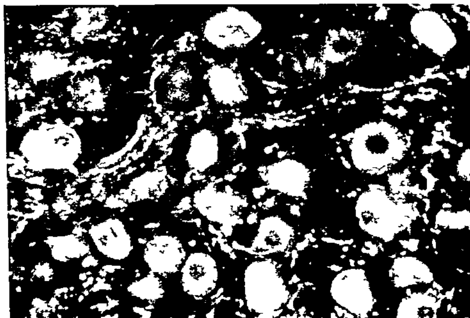


Fig 6 Inf mesent gangl cat untreated Between the fluorescent (adrenergic) nerve cell bodies are seen intensely fluorescent varico e terminals forming a ba ketlike synaptic structure around one cell (↗) × 3 0

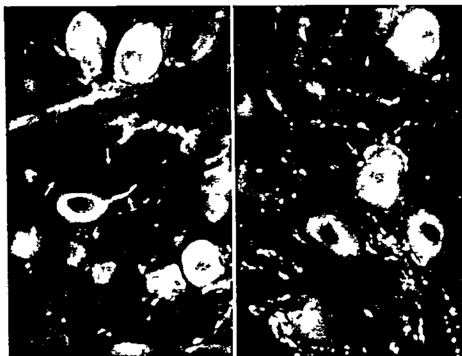


Fig 7 Sup cerv gangl rat untreated Note the n n fluorescent nerve cell bodie ( ) and th larg ell pr x (↗) lying in close connection with one of these 460

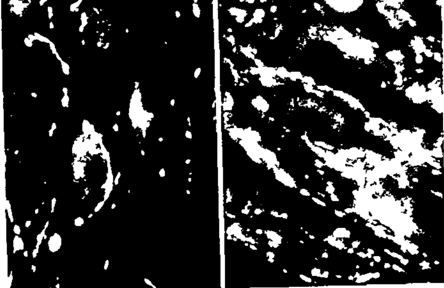


Fig 9 Inf mesent gangl cat untreated ×550
Fig 10 Inf mesent gangl cat untreated ×580

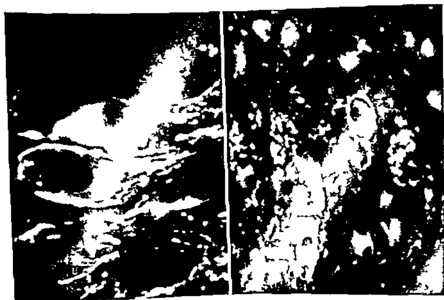


Fig 11 Celia gangl at untreated ×460
Fig 1 Cel ac gangl rabbit untreated × _ 5
Among the fluorescent (adrenergi) nerve cell bod es are seen inten ly fluore scent adren gic terminals forming here and there basketlike synaptic structures a oun l the cell bod e



Fig. 13. Submaxillary and sublingual glands of rat after DOPA (100 mg/kg i.p., 1 h). Submaxillary gland (A) with, and sublingual gland (B) without adrenergic innervation of the glandular cells. A large excretory duct (C) is devoid of adrenergic innervation. The parasympathetic submaxillary ganglion (D) contains only non-fluorescent cell bodies and no adrenergic terminals. Arterioles of varying size (E, F, K) have a prominent autofluorescent internal elastic membrane and a narrow layer of intensely fluorescent adrenergic terminals in the outer part of the smooth muscle layer of the media. The latter is quite devoid of adrenergic terminals. Note that even arterioles of very small diameter (longitudinal ↗ and transverse ↗ sections) have a very prominent adrenergic innervation. Bundles of nerve fibres (C, H) exhibit considerable fluorescence owing to an increased amine content

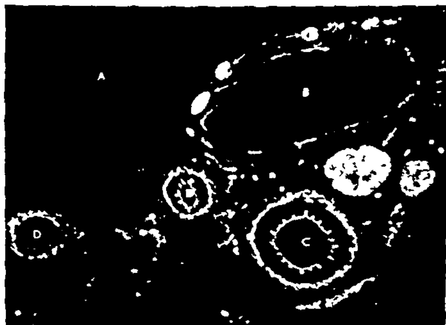


Fig 14 Sublingual gland rat untreated Sublingual gland (A) with no general adrenergic innervation of the glandular cells A venule (B) has bundles of faintly fluorescent non terminal axons ( ) in the wall Arterioles (C D) with autofluorescent internal elastic membranes and intensely fluorescent adrenergic terminals on the outside of the media A bundle of faintly fluorescent non terminal axons (↗) Note also the mast cell (↙) 1 0

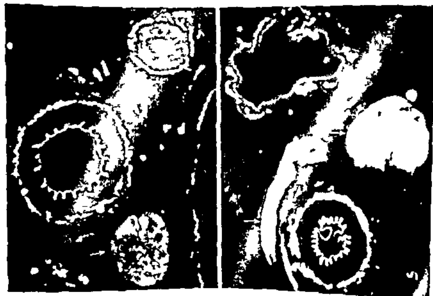


Fig 15 Submaxillary gland rat untreated ×180 30 sec exp time

Fig 16 Submaxillary gland rat L DOPA 50 mg/kg i p 2 h ×180 30 sec exp time Detail from the hilum region

Note that the bundles of non terminal adrenergic axons (↗) have a much higher intensity of fluorescence in Fig 16 than in Fig 15

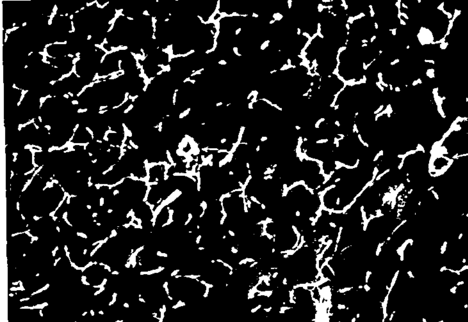


Fig 17 Submaxillary gland rat nialamide 100 mg/kg i p 5 ½ h and L DOPA 50 mg/kg i p 2 h General innervation of the acinar cells Note the two small arterioles (↗) with a very prominent adrenergic innervation × 180

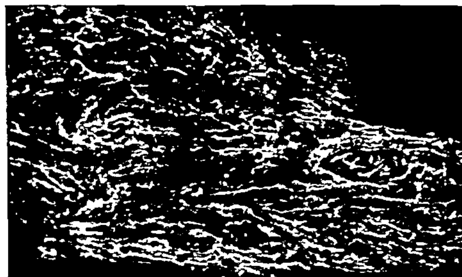


Fig 18 Sinu-auricular node of the heart rabbit untreated A dense adrenergic ground plexus with intensely fluorescent terminals is seen × 180



Fig 19 Total m unt of m senterium rat untreat d A large arteriol with an exce dingly d nse autonomic groun l pl xus with intensely fluoresc nt a lren ergic terminals an l several small r vessels with a som what less dense inner vation ×115



Fig 20 Total mount of mesente ium rat untreated An a teriole with a typical dense adrenergic ground pl xus The indi dual arico e t rminals are invisible ow ng to sl gl t liffu ion – ə

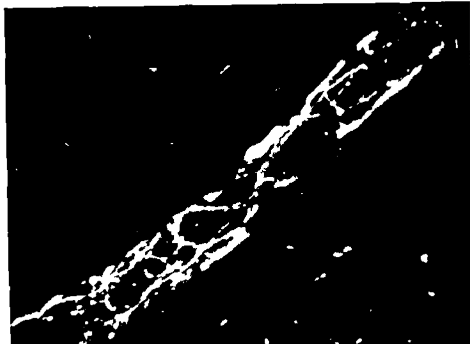


Fig. [illegible] Sublingual gland, rat, untreated. Longitudinal section of an arteriole showing the adrenergic ground plexus with one or more intensely fluorescent varicose terminals in each strand. × 400.

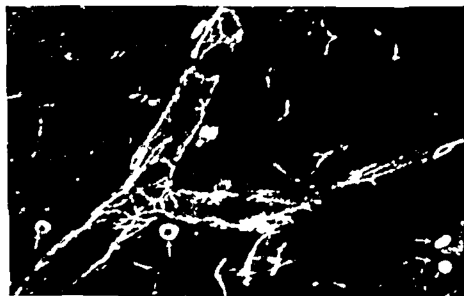


Fig. [illegible] Submaxillary gland, rat, untreated. Longitudinal section of an arteriole showing the adrenergic ground plexus. Note the mast cells (→). × [illegible]

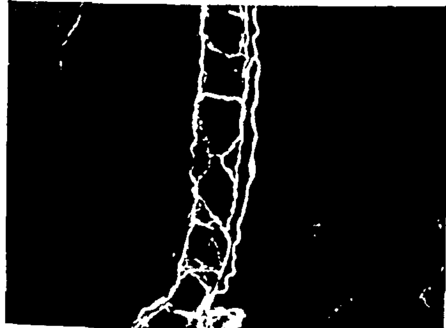


Fig 3 Wall of the cardiac region of th t mach rat untreated Longitudinal section throu h an arteriol howing th n lren rgic ground pl x



Fig 4 Prostatic es l rabbit untreated A quite d n a l n rgic innervation con ting of single intensel fluorescent varicose terminal 460



FIG. [illegible] Vas deferens from rat untreated. Circular (left) and outer longitudinal (right) muscle layers with a very dense innervation consisting mostly of single intensely fluorescent adrenergic terminals. Note the spaces ([illegible]) where terminals are arranged in a circle of about the same diameter as a smooth muscle cell. ×340



FIG 26 Vas deferens rat untreated An extremely dense innervation consisting mostly of single varicose terminals [illegible]30

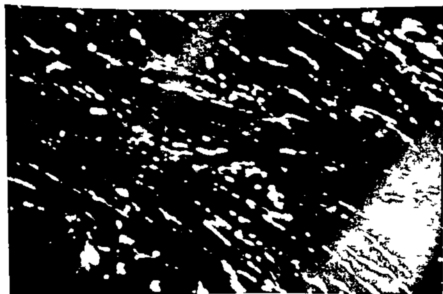


FIG 27 Vas deferens rat untreated Adrenergic terminals mainly running alone between the smooth muscle cells The diffuse light spots are terminals out of focus Focus varied during exposure ×540

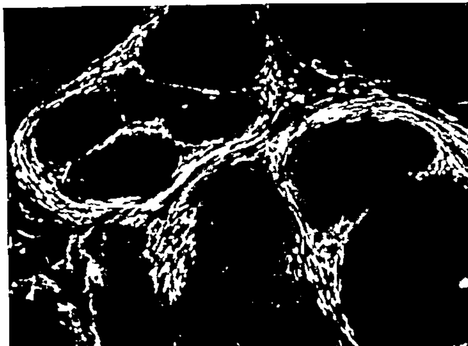
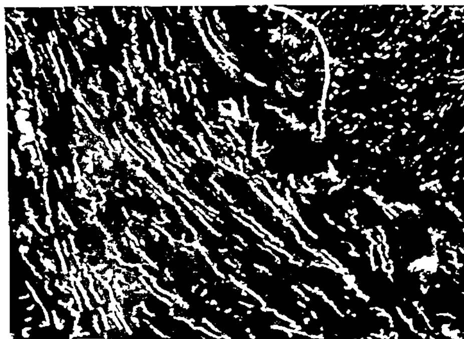


Fig 3 Pro tatic gland rat untreated Note the dense adrenergic ground plexus × 180



Fic 33 Bla ll r wall tri ne regi n cat untreated The innervation of the smooth muscl layer in th trig r reg n is quite rich but n t nearly as rich a in the va l f ren 11

Uppsala 1964. Almqvist & Wiksells Boktryckeri AB [illegible]

STUDIES ON

5-HYDROXYTRYPTAMINE STORES

IN PINEAL GLAND OF RAT

BY

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STUDIES ON 5-HYDROXYTRYPTAMINE STORES IN PINEAL GLAND OF RAT

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5 Hydroxytryptamine (5 HT) occurs in exceptionally large amounts in the rat pineal gland (QUAY and HALEVY 1962, BERTLER *et al* 1963 QUAY 1963 a) Neither electron microscopic (PELLEGRINO DE IRALDI and DE ROBERTIS 1963 DE ROBERTIS 1964) nor conventional histochemical methods (PROP and ARIËNS KAPPERS 1961) have so far been able to reveal the cellular localization of pineal 5 HT A highly specific and sensitive fluorescence microscopic method (FALCK 1962 FALCK *et al* 1962) for demonstrating certain biogenic monoamines has however, proved capable of detecting the presence of 5 HT both in the pineal sympathetic nerves and in the parenchyma (BERTLER *et al* 1963, OWMAN 1963) *In the rat a unique feature of the sympathetic nerves supplying the pineal gland was observed* namely that they contain both a catecholamine and a tryptamine (OWMAN 1964 a) 5 HT in the pineal gland of the rat follows a distinct circadian rhythm of considerable amplitude and this rhythm can be modified by changing environmental photoperiods (QUAY 1963 a) The types of the monoamines present in the pineal and their distribution between nerves and parenchyma vary widely from species to species (OWMAN 1964 b)

Much attention has been given to melatonin (N acetyl 5 methoxytryptamine), which forms on N acetylation and 0-methylation of 5 HT (WEISSBACH *et al* 1960, AXELROD and WEISSBACH 1960) The enzyme responsible for the latter step in the reaction (hydroxyindole 0 methyltransferase) has so far been found only in the pineal gland (AXELROD *et al* 1961) Melatonin has recently been studied with the assumption that it possesses hormonal activity in the mammal (ARIËNS KAPPERS 1962 BASCHIERI *et al* 1963 TILSTRA and PROP 1963, WURTMAN *et al* 1963 a) Melatonin whose synthesis is influenced by light probably *via* the sympathetic nervous system (WURTMAN *et al* 1963 b, 1964) also shows a diurnal rhythm (QUAY 1964)

Other possible metabolic pathways of pineal 5 HT is the direct 0 methylation (AXELROD and WEISSBACH 1960) and by the formation of acid metabolites through monoamine oxidase (WURTMAN *et al* 1963 b, HAKANSON and OWMAN 1964) The diurnal variation of the 5 hydroxy indole acetic acid formed in the pineal is discussed by QUAY (1964) Finally, a very high concentration of decarboxylating enzyme has recently been demonstrated in the rat pineal (HAKANSON and OWMAN 1964)

The data and considerations outlined above indicate the central position of 5 HT in studies on pineal amine metabolism This investigation presents a

detailed account of the cellular localization of 5 HT in the rat pineal gland, and of changes in nervous and pineal 5 HT following denervation of the gland and treatment of the animals with various drugs

MATERIAL AND METHODS

Fluorescence microscopic investigations — The material consists of adult rats of both sexes, 10 animals being used in each experiment Investigations were performed from October to June The following experimental groups were studied

1 Normal untreated animals The solvents for the drugs mentioned below were not injected such injections having no demonstrable effect (CARLSSON *et al* 1962 DAHLSTRÖM and FUXE 1964)
2 *Bilateral cervical sympathectomy by removal of the superior cervical ganglia* under light ether anesthesia Animals killed 5—7 days postoperatively
3 Reserpine (Serpasil, Ciba) *s c* in a single dose 0 15 0 5, 1 5 and 5 mg/kg or 1 mg/kg daily on four consecutive days The animals were killed 24 hours after the last dose Further some animals received a single dose of 5 mg/kg and were killed 1 1 ½ 3 and 5 hours later
4 Reserpine *s c* (5 mg/kg) and 24 hours later one injection of nialamide (Niamid Pfizer) (500 mg/kg *i p*) or reserpine (0 2 mg/kg) followed 24 hours later by two injections of nialamide (100 mg/kg and 200 mg/kg) with 24 hours interval The animals were killed 6 hours after the last injection
5 *a* Methyl m tyrosine (*a* MMT) in a single dose of 400 mg/kg *i p* The animals were killed after 22 hours
6 Metaraminol (Aramine Merck Sharp & Dohme) as one dose (25 mg/kg *i p*) to animals killed 5—6 hours after the injection
7 Noradrenaline as a slow *i p* administration of 5 mg/kg The animals were killed 30 min after the end of the injection

Nialamide was dissolved in saline to a final concentration of 20 mg/ml The solution was warmed to 50—60° C after which a few drops of polyethylene glycol and 1 N hydrochloric acid were added After complete dissolution of the substance the solution was neutralized by 1 N sodium hydroxide to give a final pH of 5—6 Dl *a* MMT monohydrate (Regis Chemical Company), was dissolved in saline to a final concentration of 20 mg/ml The dl noradrenaline (Ciba) was dissolved to 10 mg/ml in saline with a few drops of 1 N hydrochloric acid (final pH about 5) The solutions were prepared immediately before use

All animals were killed by decapitation under light ether anesthesia between 10 a m and 3 p m No corrections were made for variation in day length during the experimental period (6 a m —5 p m to 3 a m —9 p m) Immediately after the animals had been killed the pineal gland was dissected out together with surrounding meningeal and sometimes also epithalamic tissues The pre-

parations were frozen at once in propane cooled with liquid nitrogen and then freeze-dried and treated in formaldehyde gas to convert the biogenic amines into fluorescent compounds Serial tissue sections 4 μ thick were prepared for fluorescence microscopical analysis. All methodological details have been reported elsewhere (FALCK 1962 FALCK and OWMAN 1964)

The method is based upon the principle (FALCK et al 1962) that certain catecholamines and tryptamines in the presence of protein condense into intensely fluorescent derivatives after treatment with formaldehyde gas from paraformaldehyde In freeze-dried tissues, the reaction can be carried out without diffusion or extraction of the monoamines from their tissue stores The method allows within certain limits distinction between some of the monoamines Thus the fluorophores of the catecholamines emit a green light which in the case of adrenaline reaches its maximal intensity only after considerably longer treatment than that necessary for primary catecholamines The fluorophore of 5-HT on the other hand emits a characteristic yellow light

Formaldehyde treated preparations from heart and/or vas deferens served as technical and experimental controls Some pineal material treated as above but without formaldehyde was studied for unspecific background fluorescence and autofluorescent material.

Examination of widely different tissues from both vertebrates and invertebrates has so far revealed only 4 biogenic monoamines namely noradrenaline dopamine adrenaline and 5 HT (cf FALCK 1964 OWMAN and FALCK 1964) Though 5 hydroxytryptophan possesses the molecular properties (FALCK et al 1962 CORRODI and HILLARP 1963) necessary to form a fluorophore with formaldehyde the amounts in which it occurs in the rat pineal (QUAY 1963 b) are apparently not large enough to develop a fluorescence sufficient to interfere with that of 5 HT Melatonin which also occurs in the rat pineal gland (see below) does not exhibit any fluorescence in model systems (HILLARP personal communication CORRODI and HILLARP 1963)

Fluorimetric determinations and paper chromatography — Adult rats of both sexes were used Several groups of animals were subjected to the same procedures as those in the previous section and were compared with normal untreated animals (Table I)

Immediately after decapitation of the animals the pineal gland was dissected out and carefully freed from adhering tissues which were found to carry numerous 5 HT containing mast cells The glands were immediately put into glass stoppered beakers (8—18 glands were used in each sample) containing 2 ml 0 4 N perchloric acid The specimens were homogenized with a glass homogenizer which was later washed with another 2 ml of perchloric acid The extract obtained was centrifuged at 0° C at about 15 000 $\times g$ for 10 min and the supernatant decanted for determination of 5 HT

The method used for determining 5 HT has been described by BERTLER (1961) The amine was separated from interfering substances such as mela

TABLE 1 *Pineal glands from normal and experimentally treated animals Fluorescence intensity of pineal nerves and parenchyma and of nerves of control organs is expressed in arbitrary units from 0 to ++++ each step indicates a clearly visible change in fluorescence intensity Using the t test significant differences in pineal 5 HT levels between normal and experimental groups are marked by * for 0.01<P<0.05 ** for 0.001<P<0.01 and *** for P<0.001*

Pineal glands		Nerves of control organs (green)	Experimental groups	Pineal 5 HT Mean ± SE (n) ng / pineal	Decrease in pineal 5 HT (%)
Nerves (yellow)	Parenchyma (yellow)				
+++	+++	+++	Normal untreated animals[1]	97.0 ± 8.6 (11)	0
(+)	+++	(+)	Reserpine 0.15 mg/kg—24 hr	52.0 ± 7.9 (3)	16**
0	++(+)	0	Reserpine 5 mg/kg—24 hr	[illegible] ± 8.9 (4)	16***
(+) or 0	++(+)	(+) or 0	Reserpine 5 mg/kg—90 min	50.0 ± 0 (3)	18***
+++[2]	+++	++++	Noradrenaline 5 mg/kg—30 min	19.0 ± 5.1 (5)	19***
0	++	0	Reserpine 1 mg/kg/day × 4	38.7 ± 4.9 (5)	60***
0	++	0	Bilat cerv sympathectomy	33.8 ± 2.3 (4)	65***
+++	+++	+++	Normal untreated animals[3]	64.7 ± 10.4 (3)	0
++	+++	(+) or 0	α Methyl m tyrosine 100 mg/kg—22 hr	62.4 ± 11.3 (5)	4
0	++ or ++++[4]	0	Metaraminol 25 mg/kg—5 to 6 hr	9.5 ± 1.4 (8)	85***

[1] Animals in this and the following six groups killed at 10 a m —11 a m
[2] Green fluorescence
[3] Animals in this and the following two groups killed at 3 p m
[4] Green to yellow green fluorescence

tonin on a cation exchange resin (Amberlite XE 64) The final determination was carried out according to BOGDANSKI *et al* (1956) the fluorescence being read at 316 mμ activating and 565 mμ fluorescent wave length (uncorrected instrumental values) and at a pH corresponding to 3 N hydrochloric acid Under these circumstances melatonin exhibits a fluorescence similar to that of 5 HT, but only about one fifth as strong

For paper chromatography 10 rat pineals were extracted in 1 ml of a mixture of ethanol and redestilled water (4 1) After homogenization of the glands the extract was centrifuged and the clear supernatant decanted and evaporated *in vacuo* to a small volume, which was put on paper (Whatman 1) The chromatogram was developed descending in n butanol acetic acid water (60 15 25) (SMITH 1958) for about 6 hrs After having dried it was sprayed with a 0 5 % potassium ferricyanide solution and 10 min later with a solution of 0 5 % ferric chloride

The values obtained for pineal 5 HT content normally and after various treatments were treated statistically and compared The Student s t test was used

RESULTS

1 *Normal animals* — Fluorescence microscopy of the normal rat pineal gland (*cf* BERTLER *et al* 1963 OWMAN 1964 a) revealed a system of delicate fibres with the same general morphology and topography as the nerves running in the autonomic ground plexus (HILLARP 1946 1959 HAKANSON and OWMAN 1964) Most of the nerve fibres appeared to form a network around the intra pineal vessels in principally the same way as adrenergic vascular nerves elsewhere in the body (*cf* FALCK 1962) The vascular nerves were densest in the very periphery of the gland where the latter is covered by a rich basketwork of pial vessels From the periphery the fibres accompanied the vessels issuing into the pineal interlobular strands enclosing also the small ramifications of the vessels Extremely thin varicose axons emerging mostly from the vascular plexuses formed an intralobular network whose meshes contained the pineal parenchymal cells The nervi conarii represent a second pathway of the pineal nerves (ARIENS KAPPERS 1960 HAKANSON and OWMAN 1964) These nerves which emit a weak fluorescence entered at the apical part of the gland in the form of two or more thick bundles of non varicose fibres and split up into smaller fascicles just inside the pineal capsule The fascicles ran either alone or accompanied the intrapineal vessels The nerves divided further to contribute in the form of highly fluorescent varicose nerve fibres both to the vascular nervous plexuses and to the parenchymal nervous system

Two features distinguished the pineal nerves from ordinary vascular and parenchymal fibres a) morphologically they were much finer and showed varicosities of irregular size interspersed at irregular intervals and b) their

fluorescence was yellow in contrast with the green fluorescence of the adrenergic vascular nerves seen in the meninges outside the pineal gland

A striking feature of the rat pineal gland was an intense yellow parenchymal fluorescence which contrasted sharply with the very weak greenish background fluorescence of surrounding tissue structures and of the epithalamic part. This pineal fluorescence was confined to the cytoplasm of the parenchymal cells, while the nuclei appeared as almost dark spots

No seasonal or diurnal (9 a.m.—3 p.m.) variations were observed in the fluorescence microscopic picture of the rat pineal gland.

The distribution of mast cells was examined with fluorescence microscopy and in sections stained with toluidine blue or Astrablau (BLOOM and KELLY 1960) They were regularly found in fairly large numbers within those parts of meninges near the pineal but rarely or in at most small numbers within the actual gland

When exposure to formaldehyde was omitted from the treatment, no fluorescence was seen in either pineal nerves or parenchyma. Only a very weak unspecific background fluorescence occurred in the gland, in the meningeal and in the epithalamic tissues

Varicose nerves emitting a green fluorescence were found in that area of the meninges near the gland These nerves formed fairly dense meshworks around arteries, veins and venous sinuses The nervi conarii could also be observed in the meninges They consisted mainly of smooth fibres but also of a few varicose axons (*cf* OWMAN 1964 a) The latter exhibited an intense green fluorescence while the green light emitted by the non varicose nerves was weak. After entering the apical part of the pineal gland, the nervi conarii turned fairly abruptly yellow The intrapineal ramifications of the nervi conarii thus emitted a yellow fluorescence. This fluorescence was not an effect due to the yellow parenchymal fluorescence since the yellow colour was quite evident also in those instances where the nerves were running for a short distance in the interlobular strands which lack parenchymal cells Also most nerve plexuses around the pial vessels in the capsule of the gland exhibited a yellow fluorescence but some of them fluoresced green. When pial vessels surrounded by green fluorescent nerve plexuses entered the gland the nervous fluorescence changed into yellow from the capsular border on

The fluorophores of the monoamines are not stable in UV light. However there is a striking difference in their sensitivity to the UV light thus while the yellow fluorescence of 5-HT decreases rapidly within 1—2 min and then slowly disappear almost completely within a further 10 min the green catecholamine fluorescence does not begin to disappear appreciably until after about 30 min Therefore when the pineal sections were left in the fluorescence microscope the intense yellow parenchymal fluorescence rapidly faded under the influence of the UV light and finally disappeared During this short period little or no change was noted in the green fluorescent nerves in the pineal vicinity but the yellow fluorescence in the intrapineal nerve fibres faded

and disappeared However a faint green fluorescence characteristic of primary catecholamines persisted in these nerves

If freeze dried tissues are treated in formaldehyde gas saturated with water the catecholamines will diffuse away from their cellular sites before their condensation with formaldehyde and binding to the protein have time to occur (FALCK 1962) This is not so pronounced in the case of 5 HT Only after prolonged exposure of tissues to water saturated formaldehyde gas will the fluorophore of 5 HT slowly diffuse These circumstances were utilized in the further study of the nature of the amines in the pineal nerves Pineal glands of rats were treated at room temperature with formaldehyde gas from 40 % formaldehyde solution in glass-stoppered vessels for 10 20 and 30 min The specimens were then dried in a desiccator over phosphorus pentoxide for 3 hrs and embedded in paraffin After 10 min formaldehyde treatment there was hardly any fluorescence of adrenergic varicose nerves outside the pineal gland only very few green fluorescent bundles—but no individual fibres—then being detectable The mast cells in the vicinity of the gland exhibited a yellow or sometimes green yellow granular fluorescence The intrapineal nerves showed a green yellow to yellow fluorescence They were not quite so distinct as in the ordinarily treated specimens but the varicose fibres could clearly be observed The parenchymal fluorescence did not differ from that of preparations exposed to dry formaldehyde gas After 20 and 30 min no adrenergic nerves were seen extrapineally The mast cells as well as the nerves within the pineal gland exhibited a clear yellow fluorescence The nerves were less distinct than after 10 min treatment and only a small number of typical varicose fibres could now be observed A slight diffusion of the fluorophore of the mast cells had occurred as evidenced by a yellow halo around the cells No demonstrable change in the parenchymal fluorescence had occurred

The normal pineal gland obtained during October to June was found to contain considerable amounts of 5 HT ranging from 66 4 to 149 1 ng per pineal (when the animals were killed between 10 a m and 11 a m) and from 46 7 to 82 0 ng per pineal when they were killed at 3 a m ($P < 0\ 05$)

On paper chromatograms a large blue spot with the same R_f value as authentic 5 HT (0 45) applied as reference appeared after spraying with the two reagents described above Another but very small blue spot corresponding to melatonin ($R_f = 0\ 80$) was also identified

2 *Cervical sympathectomy* — Bilateral removal of the superior cervical ganglia caused the fluorescence to disappear in all pineal nerves as well as in the nerves running in the meninges outside the organ A slight but distinct decrease was noted in the yellow parenchymal fluorescence The pineal 5 HT was found to have decreased by 65 % ($P < 0\ 001$)

3 *Reserpine* — The pineal gland was studied after 24 hours using four different dose levels of reserpine When 0 15 mg/kg was injected only faintly yellow fluorescent nerves remained in the gland Also the green fluorescent nerves in the meninges emitted but a faint light. No definite decrease could be

observed in the parenchymal fluorescence The treatment caused a 46 % decrease ($P < 0\,01$) in pineal 5 HT

After 0 5 1 5 or 5 mg/kg no fluorescence remained in any of the nerves in the preparation The parenchymal fluorescence showed only a slight or no decrease Chemical estimation of 5 HT following the administration of 5 mg/kg reserpine showed a decrease of 46 % ($P < 0\,001$)

In another series the pineals were analyzed at four intervals after the injection of a fixed reserpine dose of 5 mg/kg to the animals Already after 1 and 1 ½ hrs the fluorescence in the nerves was severely decreased so that no or only a few faintly fluorescent axons remained in the pineal and in the meningeal tissue The parenchymal fluorescence showed little or no reduction Chemically pineal 5 HT was lowered by 48 % ($P < 0\,001$) 1 ½ hours after the reserpine injection No fluorescent nerves were found 3 and 5 hrs after the administration and the intensity of the parenchymal fluorescence was slightly decreased

Repeated reserpine injections (a daily dose of 1 mg/kg on four consecutive days) caused the fluorescence in all nerves to disappear completely By this treatment it seemed possible to obtain a somewhat more distinct reduction in the parenchymal fluorescence than before This assumption was evidenced by a lowered fluorescence which was especially obvious in the perinuclear zone Accordingly a fall in pineal 5 HT of 60 % ($P < 0\,001$) could be registered

4 *Reserpine + nialamide* — It is known that reserpine induced depletion in brain 5 HT can be reversed by the administration of nialamide while the catecholamine levels remain low (CARLSSON *et al* 1960 1962 CARLSSON 1964)

When the animals were given a large dose of reserpine followed by one nialamide injection no recovery could be noted in the fluorescence of the nerves Also the parenchymal fluorescence appeared in the same way as when treated by reserpine alone However when the animals were given reserpine in a dose just large enough to cause the nervous fluorescence to disappear (see above Section 3) and then 2 injections of nialamide the intensity of the yellow fluorescent pineal nerves was even higher than normally The same effect can be obtained by nialamide alone (BERTLER *et al* 1963) The parenchymal fluorescence was the same as in the untreated animals In the control organs on the other hand the adrenergic nerves still emitted only a slight—if any—fluorescence

5 *a Methyl m tyrosine* — A slight but clear decrease occurred in the fluorescence intensity of the intrapineal nerves The parenchymal fluorescence remained unchanged Only few adrenergic nerves showing but a very slight green fluorescence could be observed outside the pineal gland and in the heart Vas deferens contained many though only faintly fluorescent axons Chemical determinations of pineal 5 HT did not reveal any significant reduction in comparison with corresponding (afternoon) values of the untreated animals

6 *Metaraminol* — This was found to be the only compound that reduced the pineal 5 HT more than after sympathectomy The pineal nerves as well as those in the control organs had lost all fluorescence The fluorescence in the parenchyma varied from a clear decrease to considerable increase but it always exhibited a yellow green to green light It has been found that under the conditions used the drug itself emits a green fluorescence (for further discussion see OWMAN 1964 b) which was thus responsible for most of the fluorescence seen in the pineal The 5 HT in the gland was 85 % lower than the normal afternoon values ($P < 0.001$)

7 *Noradrenaline* — In pineal glands extirpated shortly after the intraperitoneal administration of noradrenaline the nerves no longer exhibited a yellow fluorescence but a strong green one having the characteristics of a primary catecholamine The nerves outside the pineal gland showed an increased fluorescence both in the smooth fibres of the nervi conarii and in the varicose terminals related to the meningeal vessels No appreciable change was noted in the colour or intensity of the fluorescence in the pinal parenchyma The 5 HT content of the pineal had decreased by 49 % ($P < 0.001$)

The histochemical appearance together with chemically determined 5 HT levels of rat pineals normally and after the various treatments is summarized in Table I

DISCUSSION

The fluorescence method has been utilized in the study of an extensive number of different tissues from vertebrates and invertebrates (*e g* CARLSSON *et al* 1962 DAHL *et al* 1962 FALCK 1962 FALCK *et al* 1963 HAMBERGER and NORBERG 1963 BERTLER *et al* 1964 CARLSSON *et al* 1964 DAHLSTROM and FUXE 1964 OWMAN 1964 b) So far the reaction conditions under which the fluorescence develops and the properties of the fluorophores have proved the same as for biogenic monoamines in model systems (pure substances in dry protein film) Final proof of the identity of the substances visualized by the histochemical method has been obtained in various tissues by parallel fluorescence microscopic and chemical analyses of normal tissues as well as of tissues of animals denervated or treated with drugs causing more or less selective depletion of monoamines From these data and from investigations of the chemical background of the reaction (FALCK *et al* 1962 CORRODI and HILLARP 1963 1964) it appears that the method in itself is highly specific for certain catecholamines and tryptamine derivatives

The fluorescence in the fibres of the pineal gland (as well as in the meninges) disappears after removal of the superior cervical ganglia (see also BERTLER *et al* 1963) which causes almost complete denervation of the gland (ARIENS KAPPERS 1960 HAKANSON and OWMAN 1964) The fibres are thus sympathetic axons arising from the superior cervical ganglia (OWMAN 1964 a)

Reserpine in doses that cause a depletion of catecholamines and 5 HT from nervous tissues (CARLSSON *et al* 1957 SHORE *et al* 1957) completely abolish the fluorescence of the pineal nerves as well as of the adrenergic sympathetic *nerves outside the gland The fluorescence is severely decreased* within 24 hours already at a dose level of 0 15 mg/kg The depletion proceeds very rapidly occurring within 1 hr of a large dose (5 mg/kg) as is also the case in central adrenergic neurons (*cf* CARLSSON *et al* 1962) *Inhibition of the* monoamine oxidase with malamide (*cf* HAKANSON and OWMAN 1964) enhances the intensity of the fluorescence in the pineal nerves (BERTLER *et al* 1963, FALCK *et al* 1964) This is in agreement with the fact that monoamine oxidase inhibitors cause a considerable rise in the monoamine levels in the brain (CARLSSON *et al* 1960) There is thus no doubt that the sympathetic pineal nerves contain a monoamine

The yellow fluorescence of the pineal nerves its characteristic UV lability, and the finding that only little diffusion of the fluorophore can be seen in the nerves when the reaction is performed in a humid milieu strongly indicate the presence of a tryptamine derivative When *a* MMT is injected in doses that cause marked reduction of the fluorescence of the noradrenergic nerves in the heart and vas deferens only a slight decrease is observed in the pineal nerves without affecting the 5 HT content measured after 22 hrs (see below) This is in good accordance with the findings (HESS *et al* 1960 PORTER *et al* 1960 CARLSSON and LINDQVIST 1962) that *a* MMT causes but a slight and transient drop in brain 5 HT while noradrenaline disappears almost completely to remain at low levels during a longer period these amines are now known to be located intraneuronally in the central nervous system (CARLSSON *et al* 1962 1964 DAHLSTROM and FUXE 1964) Finally the yellow fluorescence of *the pineal nerves but not the green fluorescence of the meningeal fibres* could be seen to reaccumulate when malamide was administered to reserpinized animals Reserpine causes a depletion of brain monoamines and inhibition of *the monoamine oxidase brings about a considerable reaccumulation of 5 HT* without affecting the catecholamine levels (CARLSSON *et al* 1960, 1962, CARLSSON 1964 DAHLSTROM and FUXE 1964) There are thus many reasons *for ascribing the yellow fluorescence in the pineal nerves to 5 HT*

The aforementioned histochemical criteria for the type of monoamine in the nerves hold also for the yellow fluorescence of the parenchymal cells 5 HT is *most probably responsible for the bulk—if not all—of the yellow fluorescence* there Of the biogenic amines known to occur in the rat pineal gland (*cf* QUAY 1963 b) and which form yellow fluorescent products with formaldehyde *only 5 HT is present in large amounts* Like PROP and ARIENS KAPPERS (1961) and QUAY (1963 b 1964) we found that the rat pineal contains small amounts of melatonin The gland from different species also contains histamine in varying amounts (GIARMAN and DAY 1958 MACHADO *et al* 1963) MACHADO *et al* (1963) could not detect any histamine in the rat pineal gland In species in which the gland did contain histamine the amounts of the latter tended to be

correlated with the number of mast cells in the gland In this connecti should be noted that only few or no mast cells were found in the rat pine the present investigation However neither melatonin nor histamine fluorescent condensation products with formaldehyde under the present co tions This has been confirmed in model systems with the pure amine HILLARP (personal communication)

The fluorimetric determinations revealed high concentrations of a pro with the same fluorescence characteristics as 5 HT Before the final determ tion the pineal extract was carefully purified by means of an ion excha The identity of the substance estimated is further strengthened by the re obtained in experiments with paper chromatography The difference betw 5 HT level at mid day and in the afternoon is presumably due to the circa variations in pineal 5 HT the levels being in good agreement with obtained by QUAY (1963 a) PELLEGRINO DE IRALDI *et al* (1963), on the o hand reported values 85 % lower for the rat pineal Although these inves tors did not report at what time of the day the animals were killed the that they used a biological assay method and obviously crude extract seem account for this discrepancy especially since the concentration of 5 HT found in the rat brain was about 60 % lower than that found by many o investigators *e g* HELLER *et al* (1962) WERDINIUS (1962) and BOGDA *et al* (1963) in fluorimetric determinations on purified extracts

One group of experiments caused distinct changes only in the pineal ne Thus noradrenaline infusion and reserpinization up to 24 hrs resulted total or almost total abolishment of the yellow fluorescence in the nerves little or no changes in the parenchymal fluorescence Pineal 5 HT decreased by 46—49 % Another group exhibited distinct changes both in nerves and the parenchyma reserpine treatment during 4 days and bila cervical sympathectomy totally abolished the nervous fluorescence and caused a slight but clear decrease in the parenchymal fluorescence inter Pineal 5 HT was reduced by 60—65 % The last mentioned experim demonstrate that a certain amount of the parenchymal 5 HT is lost by de vation From the above figures the magnitude of this pool can be rou estimated to 11—19 % of total pineal 5 HT This finds support in prelimi experiments (BERTLER FALCK and OWMAN unpublished observations) which bilateral preganglionic cervical sympathectomy was performed in resulting in a decrease of pineal 5 HT by 16 % ($P < 0\,01$)

The quantitative distribution of pineal 5 HT between pineal nerves parenchyma has been discussed preliminarily in earlier reports (BERTLER e 1963 OWMAN 1963 1964 a) PELLEGRINO DE IRALDI *et al* (1963) stated about half of pineal 5 HT is located in the nerves and the rest in the pa chyma But they did not consider the possibility that the 5 HT might b been lost only secondarily to denervation of the parenchymal cells In part of the pineal 5 HT appears to be lost in that way Moreover the a conclusion can be drawn only if the decrease in 5 HT can be directly der

strated in one or the other cell system This is not possible in the electron microscope (Pellegrino de Iraldi and de Robertis 1963), but has directly been established in the present as well as in our earlier paper (Bertler *et al* 1963)

The rat pineal gland has been found to contain small amounts of noradrenaline—about 0 3 μg/g (Potter and Axelrod 1963)—and little or no dopamine (unpublished observations) It is not possible to localize pineal noradrenaline directly in the fluorescence microscope However much suggests that the pineal noradrenaline occurs in the nerves (Owman 1964 a) After UV fading of the more labile 5 HT fluorophore a slight green fluorescence remains in the intrapineal nerves Further the nerves running to the gland show all the histochemical and pharmacological characteristics for containing a primary catecholamine There is no reason to suppose that the synthesis of the neuronal transmittor should not occur also in the terminal intrapineal parts of these nerves although they obviously take up 5 HT from the pineal cells The possibility of such an uptake is further strengthened by the recent finding that in pineal glands of new born rats the intrapineal nerves have the fluorescence characteristics of a primary catecholamine (Owman unpublished observations) while no 5 HT can be demonstrated chemically (Quay and Halevy 1962) or histochemically in the gland The same mechanism as above probably accounts for the difference in colour of the extrapineal (green) and the intrapineal (yellow) segments of the sympathetic nerves of the guinea pig (Owman 1964 b) Such an uptake mechanism is not unique for the pineal fibres for it can also be shown to occur in adrenergic sympathetic nerve fibres growing in a rat pineal gland tissue grafted to the anterior chamber of the eye (Owman 1964 a)

Thus the sympathetic nerves supplying the pineal gland of both the rat and guinea pig are remarkable in the respect that they contain two different monoamines

The slight decrease in fluorescence intensity of the pineal nerves without any change in pineal 5 HT upon treatment with *a* MMT can now be explained by a disappearance of a masked green noradrenaline fluorescence the disappearance giving the general impression of a reduced intensity This agrees well with the severe reduction seen in the fluorescence of noradrenergic nerves in the control organs

It is well established that *a* MMT selectively depletes the brain noradrenaline but causes only a slight and transient decrease in the brain stores of dopamine and 5 HT (Hess *et al* 1960 Porter *et al* 1960 Carlsson and Lindqvist 1962) *a* MMT must however be decarboxylated in the tissues to bring about a release of noradrenaline (Udenfriend and Zaltzman Nirenberg 1962) Metaraminol is a metabolite of *a* MMT that can be found in animals receiving the latter compound (Carlsson and Lindqvist 1962) it is also an active releasing agent of noradrenaline (Udenfriend and Zaltzman Nirenberg 1962) Thus it would appear that in this respect metaraminol might be as selective as its amino acid precursor *a* MMT is supposed to be

However the present findings show that metaraminol is a potent releasing agent also of the 5-HT stored in the pineal nerves and parenchymal cells.

SUMMARY

The pineal gland of the rat has been studied using a highly specific and sensitive fluorescence microscopical method for the cellular localization of certain biogenic monoamines, in combination with fluorimetric determinations of 5-hydroxytryptamine (5 HT) levels under normal and experimental conditions. Exceptionally large amounts of 5 HT have been found in the gland. This amine is located partly in the parenchymal cells and partly in the pineal sympathetic nerves. There is strong evidence to support the view that the sympathetic nerves are noradrenergic, but take up 5-HT from the parenchymal cells into their terminal portions which run within the gland. A unique feature thus exists in the sympathetic nerves of the rat's pineal in that they contain two different monoamines.

The experiments have shown that half of the pineal 5-HT is located in the sympathetic nerves and that the rest is stored in the parenchyma. A small portion of this parenchymal pool is dependent on an intact innervation and is lost on denervation. After the injection of noradrenaline the fluorescence of the pineal nerves changes to show the characteristics of a catecholamine. Half of the pineal 5 HT is then depleted showing that the neuronal 5 HT is replaced by the administered noradrenaline. No change can be registered in pineal 5 HT levels 22 hours after the administration of α methyl m tyrosine. However metaraminol causes a pronounced depletion of pineal 5 HT. A reaccumulation of the 5 HT fluorescence in the pineal nerves can be brought about by the injection of nialamide to reserpinized animals as it occurs in the cerebral nerves containing 5 HT.

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NEW ASPECTS OF THE MAMMALIAN PINEAL GLAND

BY

CHRISTER OWMAN

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NEW ASPECTS OF THE MAMMALIAN PINEAL GLAND

I. Functional Significance of Fetal Pineal Gland of Rat

II. Monoamine Stores in Mammalian Pineal Gland

BY

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The present dissertation is based upon the following papers

1 Owman Ch Secretory activity of the fetal pineal gland of the rat *Acta morph neerl scand* 1961 *3* 367—394

2 Owman Ch Pinealectomy of the rat foetus *in utero* A method for producing localized intracerebral lesions during the last third of gestation *Quart J exp Physiol* 1963 *48* 402—407

3 Owman Ch Prenatal changes in epithelium of small intestine of rat foetus pinealectomized *in utero* *Quart J exp Physiol* 1963 *48* 408—422

4 Owman Ch Further studies on prenatal functional relations between rat pineal gland and epithelium of lower ileum *Acta endocr* 1964 (in press)

5 Bertler A B Falck and Ch Owman Studies on 5 hydroxytryptamine stores in pineal gland of rat *Acta physiol scand* 1964 *63* Suppl 239

6 Owman Ch Sympathetic nerves probably storing two types of monoamines in the rat pineal gland *Int J Neuropharmacol* 1964 2 105—112

7 Owman Ch Localization of neuronal and parenchymal monoamines under normal and experimental conditions in the mammalian pineal gland In *Progress in Brain Research* Ed J Ariens Kappers and J P Schadé Elsevier Publishing Company Amsterdam 1964 *10* (in press)

They will be referred to below by Paper 1 Paper 2 *etc*

Experimental work on the function of the mammalian pineal gland was incited mainly by GUTZEIT's (1896) observation of a pineal tumor in association with precocious sexual development Further similar observations were reported and in 1910 PELIZZI described the clinical syndrome of macrogenitosoma precox These observations led MARBURG (1909) to assume that the pineal gland has some endocrine function and that the syndrome was due to the elimination, by the tumour of an inhibitory action upon sexual development However, subsequent extensive experimental investigations resulted in conflicting evidence concerning the nature of the influence—if any—of the pineal gland on sexual development (see GRUNEWALD LOWENSTEIN 1952 KITAY and ALTSCHULE 1954, MOREAU 1964) In fact perusal of the pertinent literature makes it highly unlikely that the genital abnormalities in association with pineal tumors are related specifically to the pineal gland and not, for example to the hypothalamus (*cf* DAVID *et al* 1963)

The pineal gland has also been studied extensively for any effect of for example, inhibitory nature on the thyroid and adrenal glands but no conclusive evidence has been produced (KITAY and ALTSCHULE 1954 MOREAU 1964) In a critical review of 1 672 contributions to the literature on pineal function KITAY and ALTSCHULE (1954) stated that although the available physiologic and clinical data justify the presumption that the gland is functional its functions cannot yet be defined Actually the result of some investigations indicated rather that the mammalian pineal gland possessed but little functional activity during postnatal life (*cf* IZAWA 1925 FAZIO and PERRIA 1940 BARGMANN 1943 von BARTHELD and MOLL 1954 QUAY 1958)

It thus appeared that no further advance could be expected in studies on the function of the pineal gland without new approaches and the use of new techniques Therefore the present series of investigations of the pineal gland for any functional activity during prenatal life was started in 1959 In association with such an approach it seemed of interest that in certain mammals a pineal organ is demonstrable during embryonic life but postnatally it appears reduced or is even absent (KRABBE 1920 1932, 1961) Moreover since the studies of TRETJAKOFF (1915) it is considered that in earlier phylogenesis the pineal gland shows histological signs of a secretory activity The passage of aldehyde fuchsin and chromalum hematoxylin positive material from the saccular pineal organ to the cerebrospinal fluid occurs *via* the pineal lumen in certain reptiles (STEYN 1959 see also 1958) and anura (KELLY and VAN DE KAMER 1960 OKSCHE 1960 1962) However the morphological basis for the secretion still

remains to be defined at the submicroscopic level (STEYN 1960, EAKIN et al 1963 KELLY 1962 OKSCHE and VAUPEL VON HARNACK 1963)

The present investigation has shown that pineal cells in rats during a limited period of intrauterine life contain a material with selective staining properties The material seems to be a secretory product Pinealectomy of the rat fetus in utero results in characteristic signs of deficiency The findings signify a remote action of the fetal pineal this action being limited to the period of morphologically defined secretory activity in the gland

Investigations of the mammalian pineal gland during the last few years have indicated that the organ is apparently metabolically highly active It now seems rather clear that it in fact possesses functional activity also during postnatal life although the particular function still remains to be defined (see ARIËNS KAPPERS and SCHADE 1964 MOREAU 1964)

One of the most important contributions to the understanding of pineal physiology is the demonstration of certain amines in the gland It has thus been possible for the first time to define and estimate biologically highly active compounds of known molecular configuration in the mammalian pineal gland

LERNER et al (1958 1959 1960) were the first to draw attention to the presence of amines by their discovery of melatonin (N acetyl 5 methoxytryptamine) in bovine pineal glands Melatonin is formed from 5 hydroxytryptamine (5 HT) by N acetylation (McISAAC and PAGE 1959 WEISSBACH et al 1960) and subsequent O methylation (AXELROD and WEISSBACH 1960) The enzyme, hydroxyindole O methyl transferase responsible for the latter step (AXELROD and WEISSBACH 1960) has so far been found only in the pineal gland (AXELROD et al 1961) Melatonin is metabolized by hydroxylation in the 6 position (KOPIN et al 1960) and perhaps also by the formation of acid metabolites (LERNER et al 1960)

Another amine occurring in the pineal gland namely 5 HT has also received much attention Its presence in the pineal gland was first claimed on the basis of bioassay studies on the hare by MILINE et al (1958) and in cattle by GIARMAN and DAY (1958) Later human and monkey pineals were also found to contain large but varying amounts of 5 HT (GIARMAN et al 1960) In the rat pineal 5 HT was demonstrated qualitatively by PROP (1961) and PROP and ARIËNS KAPPERS (1961) Subsequently QUAY and HALEVY (1962) found that the concentration of rat pineal 5 HT was extremely high more than twenty five times as high as that in the anterior hypothalamus

Bovine and rat pineal glands also contain small amounts of catecholamines (GIARMAN and DAY 1958 POTTER and AXELROD 1963) Finally histamine has been shown to occur in varying amounts in the pineal glands of different species (GIARMAN and DAY 1958 MACHADO et al 1963) but this amine is probably stored in mast cells (MACHADO et al 1963)

The amines and related compounds occupy a central position in current studies on the pineal gland (see further below) Knowledge of the exact cellular localization of these amines obviously provides an important basis for

the understanding of their possible function Neither conventional histochemi cal (PROP and ARIENS KAPPERS 1961) nor electron microscopic methods (PELLEGRINO DE IRALDI and DE ROBERTIS 1963, DE ROBERTIS 1964) have proved capable of revealing their storage sites in the pineal gland Recently a highly specific and sensitive fluorescence method was developed allowing the demon stration of certain monoamines at the cellular level (FALCK 1962 FALCK *et al* 1962) In the light of the above mentioned data it was obvious that the stores of some of the monoamines in the pineal gland should be evaluated using this technique

The large amounts of 5 HT in the rat pineal gland were found to be located partly in the noradrenergic sympathetic nerves and partly within the paren chymal cells In the latter a certain portion of the 5 HT appeared to be dependent upon an intact sympathetic innervation A unique condition exists in the sympathetic nerves to the pineal gland of the rat in that they contain two different monoamines The monoamines present in the pineal gland their distribution between pineal nerves and parenchyma as well as the innervation pattern of the monoaminergic nerves vary widely from species to species

PART ONE

FUNCTIONAL SIGNIFICANCE OF FETAL PINEAL GLAND OF RAT

The ontogenesis of the pineal gland in different mammalian species has re ed attention by several authors However only a few and incomplete stu (GALEOTTI 1897, FUNQUIST 1912 KRABBE 1920 SUGIURA 1935 GARDNER 1 were found to have antedated the present observations on the developme the pineal gland of the rat Moreover there was no earlier basis for the assu tion of a secretory activity in the fetal pineal gland

DEVELOPMENT OF PINEAL GLAND

(PAPER 1)

The pineal anlage of the albino rat fetus appears between 13d 15hr an days *post coitum* as a shallow evagination in the diencephalic roof in fro the posterior commissure The evagination then develops backwards to a large saccular structure over this commissure A rich vascular basketwo delicate vessels surrounds the pineal diverticulum The wide pineal lu opens towards the ventricular system In the subsequent stages the wall o diverticulum undergoes a folding process This converts the wide and reg pineal lumen into a glove like system without loss of its continuity with third ventricle During the folding process mesenchyme including bl vessels accompanies the folds along their external surface and are thu corporated within the gland As the folding proceeds the gland becom more compact formation in which the mesenchymal ingrowth forms a fr work around parenchymal lobules The organ has now by the 21 day s reached a structure resembling that of the fully developed animal In structure the lobules are smaller and more numerous and the pineal gla connected with the diencephalon by a very thin stalk

According to GARDNER (1948) pineal cells form a palisade around b vessels in 18 day embryos In favourable sections of pineal glands of c sponding age such cells were seen in palisade formations in the present inv gation but only around tubular extensions of the pineal lumen and not ar blood vessels —Simultaneously with the publication of the present results development of the rat pineal gland was outlined in a paper by ARIENS K PERS (1960) His findings agree well with those presently obtained

OCCURRENCE OF SECRETORY CELLS IN PINEAL GLAND

(PAPER 1)

The ependymal primordium forming the pineal anlage differentiates into specific pineal cells as well as into glial cells The pineal cells have a characteristic appearance The cell body located at some distance from the pineal lumen, consists of a large round to oval smoothly outlined nucleus with distinctly visible nucleoles the nucleus is surrounded by a small amount of cytoplasm From the cell body a process, 9 to 45 μ long extends towards the pineal lumen, to end at the luminal surface At the 15d 6hr stage a few pineal cells are found with their processes projecting in between the undifferentiated ependymal cells to the lumen

In 16-day fetuses the processes of some of the pineal cells are seen to contain a selectively staining granular material The material is brought out brilliantly by paraldehyde fuchsin somewhat less distinctly by chromalum hematoxylin phloxin and Bodian s silver technique and only very faintly with Alcian blue All these methods are widely used for demonstrating neurosecretion in *e g* the hypothalamo-hypophyseal system No staining occurs in control sections stained in Heidenhain s azan or in paraldehyde fuchsin without previous oxidation The selective stainability of the material its granular appearance and its location within the processes of the pineal cells were considered sufficient to warrant the assumption that the material is secretory in nature Hence these cells will be referred to as secretory cells in the following discussion

During the succeeding stages the number of secretory cells and the amount of stainable material within these cells increase At the 18 day stage the secretory cells are densely packed side by side along the numerous folds in the pineal diverticulum and the secretory material fills the whole extension of the process forming large well defined accumulations at the apical end near the pineal lumen Glial cells are interspersed among the secretory cells In subsequent stages the cell content of secretory material decreases somewhat and is then seen mainly in the apical end of the cells At 20 days *post coitum* only few secretory cells are found They are located in the pineal recess and contain only weakly staining secretory material Hardly any secretory cells are present in the rest of the gland which has by then developed into a compact structure Later on the organ is devoid of selectively staining cells except in a few cases where some cells are seen to form the wall of a "follicle trapped in the distal end of the organ

Pineal cells of similar morphology and stainability as those in the rat fetus also occurs in the pineal diverticulum of human fetuses (OLSSON 1961) Here, the cells are also incorporated in the gland when it develops into a more compact structure At that stage they no longer stain with for example paraldehyde fuchsin Thus the secretory activity as defined by selective staining seems to be limited to a short period of intrauterine life in the human fetus too

The secretory cells in the pineal gland of the rat fetus closely resemble

identically stained cells probably of secretory nature, in the saccular pineal organ of certain reptiles and anura (STEYN 1959 KELLY and VAN DE KAMER 1960 OKSCHE 1962)

ELABORATION AND RELEASE OF PINEAL SECRETORY MATERIAL

(PAPER I)

The nucleus of the pineal cells undergoes distinct changes during different stages of development of the pineal gland probably reflecting variation in functional activity Moreover certain peculiarities in the morphology of these cells have been observed lending further support to the view that the pineal cells in fact are secretory These findings together with the distribution pattern of the granular selectively stained material in the pineal cells give certain indications as to the mode of elaboration and release of the secretory material

In the early stages of pineal development the nucleus has a smooth surface a distinct membrane and a loose finely granulated chromatin structure Later on this structure becomes coarser and accumulates in groups with interspersed vacuoles Subsequently the surface of the nucleus shows peripheral indentations and in the more fully developed gland the nucleus is lobulated and contains so-called nuclear pellets (Kernkugeln) just inside the nuclear membrane These formations have often been taken as evidence of a secretory function of the pineal cells but have now turned out to be the light microscopic appear ance of nuclear invaginations or even nucleoles in various functional phases as was demonstrated in the electron microscopic study of KEVORKIAN and WESSEL (1959) In some cells the nucleus has a shrunken, pyknotic appear ance (*cf* QUAY and LEVINE 1957)

Large vacuoles are in many instances present in the process of the secretory cells The vacuoles are usually filled with very finely granular positively stain ing material In favourable sections an apically located vacuole may be seen in open communication with the pineal lumen Similar vacuoles have been observed in neurosecretory and other neurons and probably signify a high degree of functional activity (SHIMAZU *et al* 1954)

A conspicuous vesicular formation can often be seen either close to the end of a secretory process or immediately outside the cells in the pineal lumen These formations may well reflect a secretory activity because similar forma tions have been observed in other periventricular organs known to be secretory The significance of similar structures located immediately outside the pineal cells of lower vertebrates (for references see KELLY and VAN DE KAMER 1960) is disputed in electron microscopic studies A tentative suggestion of their nature has been offered in the paper by STEYN (1960)

The above mentioned features are characteristic and constant findings They are not seen in the ependymal lining of the ventricular cavities elsewhere They

may therefore be manifestations of changes in functional activity of the pineal cells

Thus the morphologic picture of the pineal cells indicates that the secretory material is formed—with active participation of the nucleus—in the cytoplasm rather than within the nucleus itself (*cf* SCHARRER and SCHARRER 1954), and that the material is transported—not extracellularly (*cf* SCHARRER and SCHARRER 1954)—but *via* the cell process towards the pineal lumen to which *it is released to reach the ventricular system The absence of selectively stained* material in the pineal lumen does not argue against such a mechanism After having been released the material may well have been carried further in the cerebrospinal fluid or with reference to the findings of HANDA and KUMAMOTO (1958) it may have lost its stainability under the influence of the ventricular fluid

To ascertain whether the selectively stained material in the fetal pineal gland of the rat has any physiological significance prenatally the pineal gland was destroyed at different stages during intrauterine life and the animals then studied for any deficiency symptoms This might help to decide whether the secretion in the pineal cells is of a non-endocrine nature as it probably is in for example the subcommissural organ (see OLSSON 1958) or whether it exerts a remote action upon some peripheral organ

METHOD FOR PINEALECTOMY OF RAT FETUS

(PAPER 2)

Perusal of the literature revealed no method for making localized lesions of small structures with reasonable reproducibility in the brain of mammalian fetuses *in utero* RAYNAUD (1943) has described a method for destroying the pituitary primordium of mice *in utero* by X ray radiation. Besides requiring a complicated apparatus the method has several disadvantages (see RAYNAUD 1959) a) the survival rate after treatment is low only about 27 % b) it is not possible to decide the moment at which destruction and arrest of function occurs in the irradiated gland c) the reproducibility is low, total and subtotal destruction being achieved only in 25 % d) the lesion is obviously quite large since the head of the irradiated fetus is reduced in size and most of the irradiated tissues are necroticized and e) it is difficult to exclude deleterious effects of irradiation even at sites remote from the central lesion

Therefore a technique was devised by which the pineal gland is destroyed by electrocoagulation According to this method the anesthetized mother animal is laporatomized and the fetal head exposed through a small incision in the uterine wall The pineal gland is situated immediately underneath the surface of the calvarium It can be well localized in relation to the meningeal veins clearly visible at this stage of development. The gland is lesioned by anodal electrolysis with a small needle electrode an indifferent electrode being placed in the uterine wall The head is replaced into the uterine lumen and the incision in the uterine and abdominal wall is sutured The earliest stage at which the operation has been performed is 15d 16½ hr *post coitum* The fetuses were removed by cesarean section at various stages before parturition (see PAPERS 3 and 4)

The rather simple method proved useful for destroying the fetal pineal gland

of the rat a) the survival rate is sufficiently high (60 %) b) the electrolysis causes marked histological changes in the area of the lesion immediately after operation c) the reproducibility is good total or subtotal pinealectomy is achieved in 64 % d) the size of the lesion can easily be adjusted Although the pineal lesion is often accompanied by inadvertent damage to nearby structures the possible effect of such extension can be checked by the use of sham operated controls in which the nearby structures but not the pineal gland are lesioned and e) the extent of the lesion can be well appreciated in histological sections

With slight modifications regarding the external mark of localization of the intracerebral structure to be lesioned and the depth of the lesion the method has successfully been used to obtain relatively well localized lesions at other sites such as the anterior and posterior hypothalamus the pituitary gland (including stalk sectioning) and the mesencephalon (OWMAN, unpublished data)

PRENATAL CHANGES AFTER PINEALECTOMY OF RAT FETUS

(PAPERS 3 AND 4)

The main purpose of the continued investigations was to study pinealectomized rats for morphologically tangible changes in any organ to disclose a possible function of the fetal gland That is the following questions should be answered

1 Do morphologically discernible changes occur in any organ in animals pinealectomized before during or after the secretory period of the gland compared with sham-operated control animals?

If so

2 What are the characteristics of such changes?
3 At which postoperative stage do the changes first appear?
4 Can the changes be ascribed to the lesion of the pineal gland or only to an effect due to operative trauma, *i.e.* do non-operated and sham-operated controls resemble one another closely with respect to the appearance and extent of changes obtained in the peripheral organ?

In a first series of experiments the pineal gland was destroyed at the 15 to 17-day stage *i.e.* immediately *before* or at the beginning of the histologically demonstrable secretory period of the gland The animals were killed near expected term Owing to lack of earlier pertinent experimental data it was not possible to predict the changes following such intervention. Many observations made during postnatal life suggest some relationship between the pineal gland and the genital sphere (*cf* KITAY and ALTSCHULE 1954) Screening of sections including the genital region of the experimental fetuses did not however disclose any overt changes ascribable to the pineal lesion —In this connection it should be mentioned that the small number of selectively staining cells occasionally seen in adult rat pineals or those pineal cells of embryonic type occur

ring in some tumors of the gland probably has no functional significance on the genital sphere, as discussed in PAPER 1 —Further analysis of the serial sections of the entire fetuses revealed marked and constantly occurring changes in the epithelium of the distal ileum in the pinealectomized animals

The epithelial cells were filled with large smooth and homogenous acidophilic globules They often crowded the entire cytoplasm leaving the nucleus only as a small compressed structure at the very base of the cell The changes differed very significantly from the picture seen in sham-operated and unoperated control fetuses in which the supranuclear cytoplasm was either devoid of inclusions or contained a few small granules only

The inclusions showed up best in sections stained by the periodic acid Schiff method The material thus stained exhibited the histochemical characteristics of a carbohydrate protein complex probably muco- or glycoprotein Material displaying the same histological and histochemical staining properties as the inclusions was present in the intestinal lumen and in the form of small spheroid bodies, in the intestinal lacteals of the lower ileum

The intestinal cells in question were of similar appearance in both the sham operated and the unoperated fetuses (PAPER 3) The changes were therefore ascribed to the destruction of the pineal gland rather than to the inadvertent lesions often occurring in surrounding structures There was no difference between the results whether the pineal was totally or only partly destroyed indicating that the function of the residual part of the gland had been interfered with also in the latter cases

There are strong reasons to believe that the intracellular corpuscles are manifestations of protein material having been absorbed—probably by way of pinocytosis (CLARK 1959)—from the amniotic fluid that is ingested by the fetus The protein is obviously absorbed in an intact state since the protein digestion mechanism is not yet developed (HILL 1956) The absorbed protein is then transported further into the circulation *via* the intestinal lacteals

The changes after pinealectomy make their first appearance at the 21-day stage *i e* after cessation of the histologically discernible functional period of the pineal gland This could be explained in different ways For example the inclusions may represent a late manifestation of some previous functional impairment of the intestinal cells or a change of the ingested material during its passage of the upper digestive tract the time lag in relation to the pineal functional period then representing the rate of passage of material along the digestive tract

When the gland was lesioned *during* the period of its secretory activity the changes in the lower ileum were not so constant and prominent in comparison with sham operated controls If the fetuses were subjected to pinealectomy immediately *after* that period the ileal epithelium appeared about the same in both these groups of pinealectomized and sham operated animals

The period during which the changes in the ileal epithelium can be evoked thus coincides with that during which large amounts of selectively staining

material occur in the pineal cells Judging from the nervous pattern of the pineal gland (ARIËNS KAPPERS 1960) it is highly unlikely that the remote effect should have been induced *via* a nervous pathway but is rather a humoral mechanism The experimental evidence lends further support to the view that the selectively stained material in the pineal cells is a secretory product

The morphologic picture of the secretory cells suggests that the secretory material is delivered to the cerebrospinal fluid and not directly to the blood stream This is a well known pathway (*cf* LOFGREN 1960 1961, 1964), from the ventricular system substances may reach other brain areas or exert effects on peripheral organs directly *via* the general circulation

PERINATAL TRANSMISSION OF NATIVE MACROPROTEINS IN RAT

The immunity from the mother to the offspring is transmitted by different routes in different species (BRAMBELL *et al* 1951) Thus in some species *e g* rabbit and guinea pig it is transferred *in utero* while in others *e g* ungulates it is passed during the immediate postnatal period by absorption of the colostrum in the gut Several animals occupy an intermediate position Thus in the rat such transfer can be demonstrated both before and after parturition (CULBERTSON 1938 1939 a and b) although the postnatal transmission is by far the greatest (HALLIDAY 1955) occurring during the first three weeks after birth (HALLIDAY 1956) Generally speaking this absorption of native proteins after birth is reflected by the presence of large acidophilic inclusions in the epithelial cells of the lower ileum (SMITH 1925 COMLINE *et al* 1951 1953 HILL 1956 CLARK 1959 PAYNE and MARSH 1962 OWMAN 1964) In rat the *small* intrauterine contribution to the passive immunity is received *in utero* from the amniotic fluid partly *via* the gut (HALLIDAY 1955 BRAMBELL and HALLIDAY 1956 VON MAYERSBACH 1958) whose epithelium then contains only small or no cellular inclusions (OWMAN 1962 PAPER 3 4 OWMAN 1964) After pinealectomy at certain stages *in utero* the intestinal epithelium absorbs proteins from the amniotic fluid (OWMAN 1962 1964) and then it contains extremely large amounts of intracellular globules

Thus by fetal pinealectomy the initiation of intact protein absorption by the gut is anticipated from postnatal to prenatal life

The significance of this apparently pronounced increase in the absorption of native proteins from the amniotic fluid to the fetal circulation is as yet unknown Some possibilities might however be considered Antigenic proteins in the amniotic fluid (LAMBOTTE 1963) might enter the fetal circulation and cross the placenta (QUINLIVAN 1964) to cause autoimmunization of the mother animal (LAMBOTTE *et al* 1963) It should be stressed that such a mechanism may perhaps be significant only in the event of a massive transfer by absorption of amniotic fluid *via* the gut of *all* fetuses present within the mother animal Another possibility is that the amniotic fluid might cause fatal

disturbances in the coagulation mechanism (Weiner and Reid 1950 Rendelstein *et al* 1951 Albrechtsen *et al* 1955 Albrechtsen 1959 Beller *et al* 1963) Or abnormally large amounts of metabolically active macroprotein fractions or active substances bound to them may be taken up from the amniotic fluid (*cf* Bangham *et al* 1961) into the fetal circulation Comparison of the figures given by Heim (1961) for fetal rat serum proteins with those of Marsh *et al* (1964) for amniotic fluid proteins does not lend support to the assumption of a normally free passage of proteins between those two pools. At least some of the serum proteins are synthetized by the fetus itself (Kelleher *et al* 1963) Finally the premature uptake of proteins by the gut may cause interference (see Morris 1964) with that protein uptake normally occurring after birth

PART TWO

MONOAMINE STORES IN MAMMALIAN PINEAL GLAND

Since the recent finding of extensive amounts of 5 HT in the pineal gland in the rat (QUAY and HALEVY 1962, BERTLER *et al* 1963 QUAY 1963 a) much interest has been focused upon this organ 5 HT is formed by decarboxylation of 5 hydroxytryptophan and the enzyme responsible for this reaction is present in large amounts in the rat pineal (HAKANSON and OWMAN 1964) High dietary levels of tryptophan—the precursor of 5 hydroxytryptophan—leads to a considerable increase in pineal 5 HT within a few days (QUAY 1963 b) The amine is metabolized along different pathways either by N acetylation with subsequent 0 methylation to melatonin (McISAAC and PAGE 1954 WEISSBACH *et al* 1960 AXELROD and WEISSBACH 1960) the enzyme for the latter step in this reaction has so far been found only in pineal tissue (AXELROD *et al* 1961) Or by direct 0 methylation of 5 HT (AXELROD and WEISSBACH 1960), or finally by formation of acid metabolites through the action of monoamine oxidase which shows high activity in the pineal gland of the rat (SMITH 1963 WURTMAN *et al* 1963 b HAKANSON and OWMAN 1964) A conspicuous circadian rhythm of pineal 5 HT (QUAY 1963 a) and such compounds as melatonin and 5 hydroxy indole acetic acid (QUAY 1964) in the gland has recently been described

METHODOLOGY

A highly specific and sensitive fluorescence microscopic method recently devised for the demonstration of certain biogenic monoamines was used in these investigations in combination with chemical determination of the monoamines in normal as well as experimentally treated animals

The fluorescence method is based upon the principle (FALCK *et al* 1962) that catecholamines and certain tryptamine derivatives included in a dry protein film condense with formaldehyde to form highly fluorescent products By means of special histotechnical procedures (FALCK 1962 FALCK and OWMAN 1964) the reaction can be performed in tissues without extraction or diffusion of the amines from their tissue storage sites

The reactions involved have been thoroughly studied in model systems with pure amines (FALCK *et al* 1962 CORRODI and HILLARP 1963 1964) Thus the primary catecholamines such as noradrenaline and dopamine condense with formaldehyde to form tetrahydro-isoquinoline derivatives which are then rapidly transformed to dihydro-isoquinolines which emit an intense *green*

fluorescence under the optical conditions used. Secondary amines such as adrenaline, also react readily with formaldehyde to form tetrahydro-isoquinolines but the second step—in this case yielding a *green fluorescent* dihydro isoquinoline with a quarternary nitrogen—requires a longer reaction time and higher temperature than the primary catecholamines. The condensation reaction with 5 HT is principally the same as that with the catecholamines, yielding a dihydroxy β carboline. However the fluorophore of 5 HT emits a *yellow light*.

In the case of the catecholamines the first step of the reaction requires the OH group in the 3 position, whereas only amines with OH groups in both 3- and 4 positions give intensely fluorescent products. Indolic compounds with a tryptamine side chain and an unsubstituted 2 position will condense with formaldehyde, those indoles with 5 hydroxy and 5 methoxy groups forming condensation products with an intense fluorescence (Falck *et al* 1962, Corrodi and Hillarp 1963, 1964, personal communication). Such amines as melatonin and histamine which occur in the pineal gland of various species (Lerner *et al* 1960, Machado *et al* 1963, Quay 1964) do not exhibit any fluorescence when treated with formaldehyde. Although such compounds as 5 hydroxytryptophan possess the molecular structure necessary to form fluorescent condensation products with formaldehyde, this indole obviously occurs in too small an amount in the gland (Quay 1963 c) to develop sufficent fluorescence to infere with that of the biogenic monoamines.

In the fluorescence microscope it is thus possible to differentiate between the catecholamines and the tryptamine derivatives, the former emit a green fluorescence while the fluorescence of the latter is yellow. Moreover the yellow fluorophore of the tryptamine derivatives is very unstable when exposed to UV light, whereas those of the catecholamines are not. It is also possible to differentiate between primary and secondary catecholamines in that they differ from one another regarding the reaction conditions necessary for development of maximal fluorescence. Another finding that has proved useful in certain cases is that the fluorophore of adrenaline, in contrast to those of noradrenaline, dopamine and 5 HT, is soluble in organic solvents such as xylene (Falck *et al* 1963). Additional support for the identity of the substances visualized in the tissues is obtained by concurrent chemical determination and fluorescence microscopic analysis of normal and denervated tissues, as well as of tissues from animals treated with drugs that cause a more or less selective depletion of the catecholamines and 5 HT.

The material was studied in a fluorescence microscope, equipped with a dark field oil condensor system. A high pressure mercury lamp (Osram HBO 200) was used as the source of exciting light. The light was activated in a Schott BG 12 (3–5 mm thick) filter having an activation peak of 410 mμ and the fluorescence light was passed through a Schott OG 4 (1 mm thick) filter, absorbing light of wavelengths below 515 mμ.

The fluorescence microscopic method has a unique sensitivity, allowing *e g*

the demonstration of noradrenaline in adrenergic neurons first done by FALCK and TORP (1962) The method has been used upon a wide variety of tissues from both vertebrate and invertebrate origin (for references, see FALCK 1964, OWMAN and FALCK 1964) The reaction conditions under which the fluorescence develops and the properties of the fluorophores have so far proved to be the same as are valid for biogenic monoamines in model systems

The pineal 5 HT has been extracted and purified using the method of BERTLER (1961) and the final determinations have been carried out according to BOGDANSKI *et al* (1956)

NEURONAL AND PARENCHYMAL MONOAMINES IN RAT PINEAL GLAND

(PAPERS 5 AND 6)

Fluorescence microscopy of the rat pineal gland reveals a system of delicate fibres with the same general morphology and topography as the nerves running in the autonomic ground plexus (HILLARP 1959) The nerves form meshworks around the intrapineal vessels and around parenchymal cells An intense yellow fluorescence is emitted from the pineal nerves as well as from the parenchymal cells Reserpinization within 24 hours and noradrenaline infusion cause the yellow fluorescence in the pineal nerves to disappear more or less completely with little or no change in the parenchymal fluorescence A simultaneous marked reduction in pineal 5 HT level is demonstrable The loss occurring is so large that it should have been observed even if it were caused by a drop in the parenchymal 5 HT Thus it is the neuronal 5 HT that has disappeared while the rest of the 5 HT resides in the parenchymal cells This assumption is corroborated by the findings obtained after bilateral cervical sympathectomy—which causes an almost total denervation of the gland (ARIENS KAPPERS 1960)—and after chronic reserpinization This is discussed in detail below

Although the fluorescent nerves disappear after bilateral superior cervical ganglionectomy this need not imply that they arise from these ganglia However bilateral preganglionic cervical sympathectomy causes no changes in the fluorescence of the pineal nerves Moreover pinealectomy—*i e* removal of the terminal portions of the nerves which causes an accumulation of fluorescent material in the fluorescent nerves proximal to the lesion (DAHLSTRÖM and FUXE 1964 a, OWMAN and SJÖSTRAND 1964)—results in enhanced fluorescence not only in the preterminal nerves to the gland but also in many cell bodies of the superior cervical ganglia Each of these ganglia contributes equally to the pineal monoaminergic nervous system with a wide overlapping from both sides as revealed by unilateral removal of the superior cervical ganglia

Although the intrapineal nerves store 5 HT they have turned out to be terminals from noradrenergic sympathetic neurons

1 Yellow fluorescent nerves have never been observed outside the pineal gland neither normally nor under conditions of increased fluorescence in nerves running to the gland e g after pinealectomy, the extraglandular segments of the pineal nerves have constantly been found to emit a green light

2 After pinealectomy, which causes an enhanced fluorescence of cell bodies of the superior cervical ganglia, the fluorescence of these cells is always green

3 It has directly been demonstrated that the pineal sympathetic nerves are capable of taking up 5-HT Firstly injection of 5 HT and a monoamine oxidase inhibitor produces a yellow fluorescence in the nerves running outside the gland, and an increased yellow fluorescence in the intraglandular parts of the pineal nerves Secondly the adrenergic nerves growing into pineal tissue transplanted to the anterior chamber of the eye emit a yellow fluorescence in those parts running within the yellow fluorescent transplant

4 α Methyl m tyrosine treatment results in an abolition of the fluorescence in the extraglandular parts of the pineal nerves but little change in the intrapineal segments when studied 22 hours after the administration

5 After exposure to UV light causing the more labile 5 HT fluorophore to fade a green fluorescence characteristic of a primary catecholamine persists in the intrapineal nerves The green fluorescence in the extraglandular segments of the nerves remains essentially unchanged Moreover, chemical analysis has revealed the existence of small amounts of noradrenaline in the gland (POTTER and AXELROD 1963)

6 Recently it has been found (OWMAN unpublished observations) that during the first two weeks of postnatal life when the rat pineal contains no chemically (QUAY and HALEVY 1962) or histochemically demonstrable 5 HT, the intrapineal nerves exhibit a green fluorescence

7 Finally it should be noted that the granulated vesicles supposed to be the submicroscopic storage site for noradrenaline in adrenergic nerves (*cf* DE ROBERTIS and PELLEGRINO DE IRALDI 1961 RICHARDSON 1962 PELLEGRINO DE IRALDI *et al* 1963 a DE ROBERTIS 1964 WHITTAKER *et al* 1964) are also found within the pineal nerve terminals (MILOFSKY 1957 1958 PANAGIOTIS 1962 WOLFE *et al* 1962 DE ROBERTIS 1964)

Thus the nerves supplying the rat pineal gland are adrenergic but take up 5 HT from the parenchyma to store it together with noradrenaline in their intrapineal parts

Certain experimental conditions i e reserpine treatment for 24 hours or infusion of noradrenaline abolish the nervous fluorescence almost selectively thus allowing an estimation of the amount of 5 HT located in either of the nervous and parenchymal stores Fluorimetric determinations revealed a loss of about half of the pineal 5 HT demonstrating that an equal distribution of 5-HT occurs between these storage sites On the other hand sympathetic denervation is followed not only by disappearance of the nervous fluorescence, but also by a slight but clear decrease in the parenchymal fluorescence fluorimetric estimation shows a further 15 % decrease in pineal 5 HT Thus the

denervation experiment indicates that a small part of the parenchymal pool is dependent upon an intact sympathetic innervation This is corroborated by recent findings that preganglionic denervation also causes a 15 % decrease in the 5-HT level of the pineal gland (BERTLER, FALCK and OWMAN unpublished observations) Preganglionic denervation is known to cause no significant change in the amine content of nervous tissues (*cf* SJOSTRAND 1962) Effects similar to those produced by postganglionic sympathetic denervation can be brought about by repeated injections of reserpine for several days That the loss of 5 HT is of the same order of magnitude as after denervation and that such drastic treatment with reserpine cannot further affect the parenchymal 5 HT strongly indicates that the reserpine effect in fact depends upon the "chemical denervation which is obviously induced If it does the reserpine induced denervation apparently requires more than 24 hours to cause a clearly visible reduction in the fluorescence intensity of the parenchymal cells

Amine stores that are refractory to reserpine occur not only in the pineal cells mast cells (MORAN and WESTERHOLM 1963) and enterochromaffin cells in the fundus region of the stomach (ZBINDEN *et al* 1957) also being fairly resistent to reserpinization Further denervation of the adrenal medulla in the rat considerably reduces the sensitivity of the medullary cells to reserpine (HILLARP 1960)

Other investigations also suggest that the pineal parenchymal cells are influenced by sympathetic denervation Thus, ARIENS KAPPERS *et al* (1964) found such intervention to be followed by an increase in pineal lipids The light induced decrease in the activity of the melatonin forming enzyme (WURTMAN *et al* 1964) and the similarly induced increase in the activity of the decarboxylase forming 5 HT (SNYDER *et al* 1964) is abolished after bilateral cervical sympathectomy

The fact that adrenergic nerves located within the pineal gland contain 5 HT points to the possibility that 5 HT as such passes out of the parenchymal cells Findings in a current investigation suggest a high turn over rate of parenchymal 5 HT Thus NSD 1015 (CLARK 1959 CARLSSON 1964) and Ro 4-4602 (PLETSCHER and GEY 1963 KUNTZ 1964), which are potent inhibitors of the decarboxylating enzyme in the rat pineal gland as shown by HAKANSON and OWMAN (1964) using direct enzyme estimations cause the fluorescence in the parenchyma, but not in the nerves to disappear completely within one hour (FALCK, OWMAN and ROSENGREN unpublished observations) The present findings suggesting a considerable turn over rate of 5 HT in the pineal parenchyma as compared with the nerves do not agree with those obtained by PELLEGRINO DE IRALDI *et al* (1963 b) They studied the increase in pineal 5 HT content upon the administration of 5 hydroxytryptophan and found it to be considerably smaller in denervated than in normal glands This difference may well be due to the loss of the sympathetic innervation necessary for the parenchymal cells to respond to 5 hydroxytryptophan with an increased 5 HT synthesis (*cf* SNYDER *et al* 1964) rather than to the loss of a high

synthetizing capacity in the pineal nerves Since DOPA decarboxylase activity decreases only little after sympathetic denervation (HAKANSON and OWMAN 1964), the 5 HT synthetizing capacity of the nerves seems to be fairly small

In some respects the response of the intraglandular segments of the pineal adrenergic nerves containing 5 HT to certain drugs resembles that of the 5 hydroxytryptaminergic nerves in the central nervous system Thus, *a* methyl m tyrosine causes only a transient depletion of the 5 HT in the pineal nerves, and after depletion by reserpine the 5 HT reaccumulates on nialamide treatment (*cf* CARLSSON *et al* 1962, DAHLSTROM and FUXE 1964 b) On the other hand metaraminol which is a metabolite of *a* methyl m tyrosine, causes a release of 5 HT stored in pineal nerves and parenchymal cells

On the basis of the present findings on the monoamine stores of the rat pineal gland it should be pointed out that *the rat pineal offers unique conditions for studies on certain monoaminergic mechanisms* the pineal nerves store two different types of monoamines one of which is supplied to the nerves from the parenchymal cells Furthermore the pineal cells synthetize and store large amounts of 5 HT but in these two processes they are dependent on an intact sympathetic innervation

PINEAL MONOAMINE STORES IN DIFFERENT SPECIES

(PAPER 7)

The monoamine stores in the pineal gland have been investigated in a number of different mammals Glands from some of these animals are often used for the preparation of extracts to be administered in experimental studies on pineal function The type of monoamines present in nerves and parenchyma, as well as the distribution of the monoaminergic nerves between pineal blood vessels and parenchymal cells differ widely from species to species

As a basic pattern the fluorescent nerves within the pineal derive from the nervi conarii and from vascular nerve plexuses supplying the meningeal vessels that enter the gland The fluorescence in the nerves is abolished by bilateral removal of the superior cervical ganglia (rat rabbit and cat) The possibility of nervous pathways between the pineal gland and the habenular and posterior commissures has been discussed by several authors (see ARIENS KAPPERS 1960) With respect to monoaminergic nerves no such relation has been found in the present series In the golden hamster rabbit cat sheep pig and cattle the pineal nerves emit a fluorescence characteristic for primary catechol amines whereas the nerves of the guinea pig are histochemically seen to contain a tryptamine derivative probably 5 HT In this animal as in the rat no yellow fluorescent but only green fluorescent nerves were found outside the pineal gland the yellow fluorescence appearing first at the entry of the nerves in the gland This and the fact that the guinea pig parenchyma emits a yellow fluorescence which seems to be due to 5 HT indicates that in the guinea pig

too the nerves supplying the pineal gland are adrenergic, but take up 5 HT into their intrapineal parts A yellowish UV labile fluorophore characteristic of a tryptamine derivative is also present in the pineal cells in the hamster In the pineal gland of the pig the parenchyma exhibits a strong yellow fluorescence, and accordingly large amounts of 5 HT were measured in the gland but in this animal as well as in the hamster, the pineal nerves emit a green fluorescence Several explanations can be offered for this difference in comparison with the rat and guinea pig pineals in which the nerves are yellow-fluorescent In the hamster and pig the parenchymal cells may not release their tryptamine derivatives to allow them to enter the intrapineal nerves, it should be noted that the cellular distribution of the yellow fluorescent material in the parenchymal cells of these animals differs distinctly from that in the rat and guinea pig probably reflecting a different monoamine storage mechanism Or the amounts of tryptamine derivative released by the parenchymal cells may not be sufficient to be taken up into the intrapineal nerves and displace the noradrenaline present in them Finally, an intense yellow fluorescence has been found also in the parenchyma of the pigeon pineal gland and the gland contains high levels of 5 HT (0 08 µg/pineal) (BERTLER LJUNGGREN and OWMAN unpublished observations) In contrast to the findings in the rat, this 5 HT store is very sensitive to reserpinization No parenchymal fluorescence occurs in the pineals from sheep cat rabbit, and cattle

There are several ways to explain why the pineal glands in some species lack parenchymal fluorescence a) the parenchymal cells simply do not synthetize a monoamine in these animals b) the amine is not accumulated in the parenchyma in amounts large enough to be visualized with the fluorescence microscopic method, c) a monoaminergic mechanism may be operating in the cells the monoamine in question not forming fluorescent derivatives with formaldehyde There is reason to believe that the parenchymal cells lacking fluorescence in these pineals are actually involved in the metabolism of monoamines Thus although the fluorescence picture of the pineal parenchyma in cow and pig pineals is quite different the glands in both animals have a high activity of decarboxylating enzyme Moreover addition of pyridoxal 5 phosphate the co enzyme for DOPA decarboxylase to the incubation medium produces a tenfold increase in the enzyme activity in these pineal glands (HAKANSON and OWMAN unpublished observations) It is also known that comparatively large amounts of catecholamines are taken up *in vitro* by both cat and bovine pineal tissue (BRODIE *et al* 1960 DENGLER *et al* 1962) It seems unlikely that this high monoamine uptake can be related only to the fluorescent structures seen in the glands from these animals

The distribution and amount of the monoaminergic pineal nerves varies considerably from species to species In the guinea pig the nerves are seen almost exclusively as rich plexuses around vessels while in the rabbit and sheep they form a rich network mainly related to the parenchymal cells The pineal nerves of the rat hamster and cat occupy an intermediary position in that they

are distributed both around vessels and in the parenchyma Only some very few isolated adrenergic nerves are found in the pig and bovine pineals It should be noted in this connection that almost all nerves in the rat pineal are monoaminergic and arise from the sympathetic nervous system (ARIËNS KAPPERS 1960) while it seems probable that the pig and bovine pineals have a rich supply of cholinergic nerves, as evidenced by the high acetylcholinesterase content of fibre structures around the vessels in the glands from these animals (ARVY 1963)

MONOAMINES AND PINEAL FUNCTION

The finding that amines and related compounds occur in the mammalian pineal gland has offered a new point of departure for studies on pineal function. Indolic compounds of different kinds originating from the pineal gland have already been assumed to influence the three target structures most studied, namely the genital sphere the thyroid gland and the adrenal cortex

Since the classical observations of McCORD and ALLEN (1917) it has been known that pineal extracts cause melanin aggregation in the melanophores of the amphibian skin This effect can now be fully explained by the presence of melatonin (LERNER *et al* 1958) in such pineal extracts (see LERNER and CASE 1959 BURGERS and VAN OORDT 1962 QUAY and BAGNARA 1964), this compound having a very potent pigment aggregating effect on amphibian melanophores (MORI and LERNER 1960) Curiously enough there is as yet no evidence that amphibian pineal organs synthetize or contain melatonin (EAKIN *et al* 1963) although there is strong reason to believe that the pineal area is involved in the amphibian body blanching by way of the melanophores (BAGNARA 1963) Paradoxically the mammalian melanocytes do not seem to change their pigment distribution on melatonin injection (LERNER and CASE 1960, LERNER personal communication) although this amine occurs in mammalian pineal glands (LERNER *et al* 1958)

It has recently been suggested that melatonin may be a pineal hormone in mammals WURTMAN *et al* (1963 a) found a delay in vaginal opening and a decrease in ovarian weight and incidence of vaginal estrus in maturing female rats receiving melatonin. However ARIËNS KAPPERS (1962) and TILSTRA and PROP (1963) obtained no effect on vaginal opening and no histological changes in spermatogenesis and oogenesis in rats given daily doses of melatonin for a long period although ARIËNS KAPPERS (1962) reported an inhibition in the growth of the seminal vesicles Recently it has been found that another O methylated pineal indole derivative—5 methoxytryptophol—has an even more marked effect than melatonin on the reduction of ovarian weight and the incidence of estrus in rats (McISAAC *et al* 1964) Finally when administered in large doses 5 HT has also been observed to cause distinct changes in the genital sphere delay of vaginal opening and onset of estrus decreased ovarian

uterine and vaginal weight in mice (ROBSON and BOTROS 1961) and similar effects on the genital organs have been obtained in rats (MOSZKOWSKA KAGAN 1963) The effect on the genital sphere could be reproduced upon treatment with monoamine oxidase inhibitors (ROBSON and BOTROS 1961)

BASCHIERI *et al* (1964) found melatonin to prevent methylthiouracil induced thyroid hyperplasia and to reduce thyroid uptake of I However a decrease in thyroid activity as measured by I[1] has also been obtained after administration of 5 HT The effect is further enhanced if the 5 HT is combined with a monoamine oxidase inhibitor (ZIZINE 1959)

Pinealectomy in the rat has been found to cause a considerable increase in the amount of neurosecretory material in the paraventricular nuclei and in the paraventricular hypophyseal tracts (MOREAU 1964) On the other hand 5 HT results in a marked decrease in the amount of the neurosecretory material in this system (KIVALO *et al* 1958)

After an adrenoglomerulotropic factor had been claimed to occur in pineal tissue (FARREL 1959) much interest was devoted to the possible stimulatory action of the pineal gland on aldosteron secretion The adrenoglomerulotropic activity was later found to be most pronounced for a couple of carbolines formed by cyclisation of melatonin (FARREL and McISAAC 1961) But it is as yet not known whether this reaction can occur *in vivo* It seems even uncertain from which tissue the adrenoglomerulotropin emanates (FARREL *et al* 1962) Moreover the pineal influence on aldosterone secretion has been seriously questioned (BUGNON and MOREAU 1962)

It was observed by ROSENKRANTZ (1959) and ROSENKRANTZ and LAFERTE (1960) that 5 HT increases the secretion from the adrenal cortex of a factor with sodium retention activity Recent *in vitro* studies have made it likely that aldosterone secretion can be stimulated by 5 HT and moreover that 5 HT well may be a specific factor in pineal glands influencing the aldosterone secretion (JOUAN 1963 JOUAN and SAMPEREZ 1964 ROMANI 1964)

This outline gives an impression of the present confused state regarding the biological significance of pineal indoles Although certain indoles can mimic some of the effects obtained with pineal extracts it is by no means clear which —if any—of these compounds constitutes the functional end product of the pineal gland

In many of the species investigated the histochemical method has directly disclosed biogenic monoamines within the pineal parenchymal cells or it has at least been possible to demonstrate the occurrence of enzymes involved in amine metabolism in the gland In the rat moreover direct evidence for a considerable monoamine turn over has been produced Finally in this species the metabolism of the parenchymal monoamine is influenced by the sympathetic innervation

FINAL REMARKS

The present findings of the secretory activity of the pineal gland prenatally on one hand and of the monoamine stores in the pineal gland postnatally on the other indicates the possibility of a two fold functional significance of the pineal gland in one and the same animal during two different phases of life Similar dual functions have been suggested to occur in the development of the pineal complex in lower vertebrates Thus in certain land living anura the pineal complex is dominated by the frontal organ probably exerting a photoreceptor function In adult stages the frontal organ is reduced, while the epiphyseal portion shows up as a well developed glandular structure (OKSCHE 1955) Similarly the pineal organ of the newt undergoes a characteristic change in cytology from highly differentiated photoreceptor like cells during larval stages into cells with morphologic signs of secretory activity culminating in adult stages This conversion probably denotes a basic shift in function of the organ (KELLY 1962 1963)

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CENTRAL NERVOUS MONOAMINES AND HORMONE INDUCED ESTRUS BEHAVIOUR IN THE SPAYED RAT

BY

BENGT J ME

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FROM THE DEPARTMENT OF PHARMACOLOGY UNIVERSITY OF UPPSALA SWEDEN
(HEAD PROF DR ERNST BÁRÁNY)

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BENGT J MEYERSON

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Contents

Introduction

It is well known that the estrus behaviour in female mammals, with cyclic periods of heat is dependent on gonadic hormones The relationship between estrus behaviour and estrogen in the female rat was studied thoroughly by Hemmingsen (1932) He showed in agreement with earlier similar experiments of Allen (1924) that ovariectomy brought about almost immediate loss of estrus behaviour, while the accepted criteria of heat could be produced by injection of estrin Several authors have described the same effects in other animals and this has been reviewed by Beach in Hormones and Behaviour (1948)

There is reason to assume that the estrus behaviour is activated by a direct effect of the estrogen on structures within the hypothalamus Harris et al (1958) showed that stilbestrol dibutyrate implantation in the posterior hypothalamus of the spayed cat resulted in complete estrus behaviour in spite of no peripheral estrus manifestations Estradiol implantation in the anterior hypothalamus activated heat response in spayed rats while implants within other parts of the hypothalamus had no such effect (Lisk, 1962)

The significance of the hypothalamus for female estrus behaviour is also indicated by the results from lesion experiments and electric recording which are reviewed by Sawyer (1960)

A possible relationship between cerebral monoamines and this hormone-activated behaviour was investigated in view of the accumulating evidence that monoamines are involved in transmitter activity within the central nervous system

The estrus behaviour in ovariectomised rats is normally activated by progesterone after pretreatment with estrogen Boling and Blandau found (1939) that in rats the female heat response is far more effectively activated by this combination of the two hormones than by estrogen treatment alone It has recently been shown that amine depletion by reserpine or tetrabenazine (Meyerson 1964 b) could be substituted for progesterone in the estrogen progesterone treatment The estrogen is still necessary Moreover increase of cerebral monoamine levels achieved by monoamine oxidase inhibitors reduced the estrogen progesterone activated heat response (Meyerson 1964 a) This led to the suggestion that monoaminergic central nervous pathways exist which mediate heat inhibition

The purpose of the present investigation was to investigate further the relation between the hormone activated estrus behaviour and the central nervous catecholamine and serotonin levels

Methods

A Experimental animals

Ovariectomised albino Sprague-Dawley rats, weighing 180—300 grams, were used They were fed commercial rat pellets (Anticimex 210, Norrvik-en Sweden) and tap water ad libitum The ovariectomy was permormed 2—3 weeks before the animals were used in experiments, the absence of estrus cycle checked by taking vaginal smears during a period of 6 days

B Estrus behaviour tests

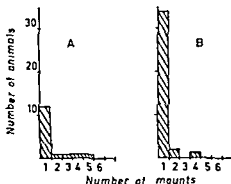
The display of lordosis response when mounted by a male was used as an indicator of estrus behaviour Other behaviour manifestations associated with heat such as acceptance of the male the female darting movements or ear wiggling were found difficult to estimate consistently at submaximal dose levels

The method used follows that earlier described (Meyerson, 1964 a) The estrus behaviour was activated by injection of estradiol benzoate and progesterone, in that order 48 hours apart The animals were kept under reversed day night rhythm (light from 9 p m —9 a m) Progesterone was administered at 8 a m the optimal response is achieved 6—8 hours later

The behaviour of each female was tested with 2 different vigorous males for a period of 1 minute each It was earlier described (Meyerson 1964 a) that sedation of the females reduced the eagerness of the male to copulate Besides using selected active males throughout the investigation this problem was overcome by the following maneuver a highly receptive female treated only with hormones was introduced into the observation cage for a few moments prior to the sedated female

Each female was offered at least five mounts per test the males were immediately changed if exhausted, to ensure that this amount was reached The frequency of mounts during the 2 minute tests only exceptionally ex ceeded 8—10 mounts The test was interrupted as soon as the female had displayed a clearcut lordosis response

Fig 1 shows the number of mounts necessary to elicit the lordosis res ponse at two dose levels of estradiol benzoate 2 5 and 25 μg/kg followed 48 hours later by 0 4 mg animal progesterone Most animals responded to the first mount



Treatment At 0 hrs	Treatment At 48 hrs	Estrus response %	Number of animals tested
A E 2 5 µg/kg	progesterone 0 4 mg/anim	27	60
B E 25 µg/kg	progesterone 0 4 mg/anim	88	42

FIGURE 1 Frequency distribution of the number of mounts necessary to elicit lordosis reflex in estradiol (E) + progesterone treated spayed rats
The animals were tested 8 hours after the progesterone treatment Six mounts were offered

C *Injected materials*

Estradiol benzoate and progesterone were dissolved in olive oil and injected in a volume of 0 20—0 30 ml

Neuropharmacological compounds used were Reserpine (Serpasil® CIBA ampoules 2 5 mg/ml) Mo 911 (N methyl N benzyl 2 propynylamine hydrochloride Pargyline® Abbot Lab) 5-HTP(DL-5 hydroxytryptophane Sandoz) DOPA (DL dihydroxyphenylalanine Hoffman La Roche), DOPS (DL threo 3 4 dihydroxyphenylserine Hassle) H 22/54 (α propyl dopacetamid Hassle)

The reserpine blank used consisted of 10 % polyethyleneglycol 400 1 % ethyl alcohol 0 2 % EDTA and 0 1 % citric acid 5 HTP was dissolved in 0 01 N HCl and the solution diluted with 9 volumes of saline The other substances were dissolved in saline

Doses mentioned in the text and tables always refer to the forms of the compounds stated above All injections were subcutaneous unless otherwise stated

D Estimation of catecholamines and serotonin

The animals were sacrificed by decapitation and the brains immediately removed Dissections were performed on ice-cold ceramic tiles

Whole brains were cut into pieces and immediately put into the homogenizer containing ice cold 0 4 N perchloric acid EDTA and ascorbic acid (see below)

In experiments where further dissection was carried out this was done in two different ways which in the following will be referred to as Method A and Method B Cerebellum, pons and medulla oblongata were excluded in both methods Fig 2 was constructed to facilitate the description of the dissection

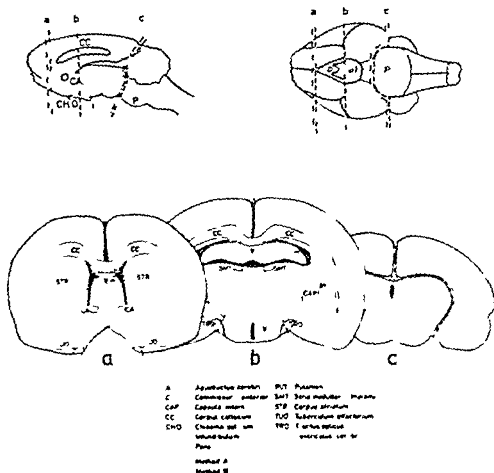


FIGURE 2 Dissection procedure used in the amine assay experiments The lower pictures show the section surfaces of the transverse sections a b and c

METHOD A In the first place the brain was placed upside down and two transverse sections were made one immediately anterior to pons (Section c in Fig 2 the separation of the two lines is only for clarity) the second anterior to the optic chiasm in the apex of the angle formed underneath the brain by the two olfactory tubercles (Section a in Fig 2) The former section was made perpendicular to the longitudinal axis of the medulla oblongata

From the upper side of the brain the lateral ventricles were reached via the sulcus centralis (see point dash line) The ventricles were followed bluntly downwards to their inferiolateral corner from where sharp dissection was made to the furrow underneath the brain formed by the borderline between the hemispheres and the diencephalon — mesencephalon region

The portion within the point dash line in Figure 2 bounded anteriorally by the transverse section a and posteriorally by c constituted one fraction (Fraction A 1) and the rest of the tissue was collected into another fraction (Fraction A 2) for separate assay

METHOD B In addition to the above mentioned transverse sections a and c a third was made half way between the optic chiasm and the infundibular stalk (Section b in Fig 2) The slices of tissue between a and b and between b and c were turned with the section surface b upwards and a vertical cut was made between the tractus opticus and striae medullares structures which are both easily recognised macroscopically (see dash line)

Three fractions were taken for separate assay Fraction B 1 was the portion within the dash line between the transverse sections a and b fraction B 2 that within the dash line between the transverse sections b and c The remaining brain tissue was collected into one portion Fraction B 3

The purpose of this dissection was to cut out the regions expected to be relevant to estrus behaviour and monoamines (A1 B1 B2)

Fraction A1 comprised the diencephalon mesencephalon and a minor part of the capsula interna

Fractions B1 and B2 comprised the medial parts of the thalamus the hypothalamus and partly the mesencephalon mainly the rostral part

By means of the fluorescence method for histochemical demonstration of monoamines (Falck 1962) the occurrence of monoamine containing neurons in the brain was recently demonstrated It was shown that the mesencephalon mainly contained cell bodies while fine varicose fibres assumed to be the terminal parts of axons were demonstrated within the hypothalamus (Carlsson Falck and Hillarp 1962 Dahlstrom and Fuxe 1964)

D Estimation of catecholamines and serotonin

The animals were sacrificed by decapitation and the brains immediately removed Dissections were performed on ice-cold ceramic tiles

Whole brains were cut into pieces and immediately put into the homo genizer containing ice cold 0 4 N perchloric acid, EDTA and ascorbic acid (see below)

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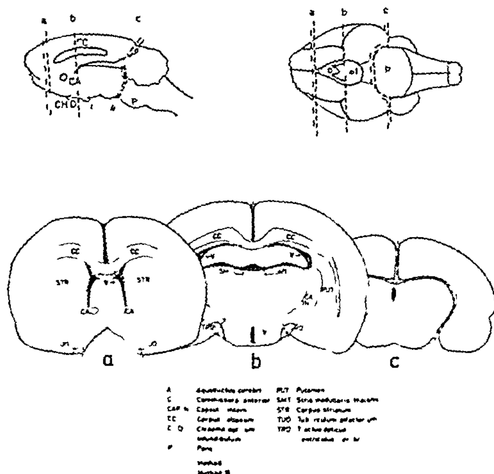


FIGURE 2 Dissection procedure used in the amine assay experiments The lower pictures show the section surfaces of the transverse sections a b and c

assay, was reduced to one half of that described by Bertler et al and the amount of water added was adjusted to yield a final volume of 5 ml

Dopamine was determined according to Carlsson and Waldeck (1958) with the modification that the amount of 0 02 N iodine solution was increased to 0 1 ml and the final pH was adjusted by 1 6 ml 5 N acetic acid (Carlsson and Lindqvist, 1962)

Serotonin

The tissue was homogenized in 2—3 ml/g 0 1 N HCl and the entire sample was immediately extracted according to the method of Bogdanski et al (1956) with the modification of the quantities of reagents given by Udenfriend (1958) However no borate buffer wash was done except when the animals had been treated with 5 hydroxytryptophane Preexperimental results revealed the same serotonin values with and without this wash

*

The fluorescence was read in an Aminco Bowman spectrophotofluorometer equipped with an Osram XBO lamp After rearrangement to corresponding fluorophores the activation and fluoroscence maxima for authentic noradrenaline (L noradrenaline D bitartrate monohydrate N V Philips Duphar, Amsterdam) dopamine (3 hydroxy tyramine hydrochloride Hoffman La Roche Basle) adrenaline (L adrenaline bitartrate Rhone Poulenc Paris) and serotonin (5 hydroxytryptamine creatinine sulfate Hoffman La Roche Basle) were identical with those obtained from the brain eluate

The fluorescence intensities were read at (uncorrected instrumental values, activation wave length/fluorescence wave length both in mμ) serotonin 295/545 dopamine 325/275, noradrenaline, adrenaline 400/515 and 450/515

The fluorescence of the samples was compared with a pure standard (a standard treated in the same way as the 2 ml sample of the catecholamine eluate) For serotonin the standard was treated identically with the final acid extract There was good agreement between the pure catecholamine standards and an internal standard (a standard added to a tissue eluate) No such comparison was made with serotonin

Known amounts of catecholamines and serotonin were regularly added to one half of a divided homogenate Recoveries ranged from 70—100 % with mean values about 85 % Whole brain values are evident from Table 1 The values of catecholamines and serotonin are expressed as free base throughout the investigation

TABLE 1

Levels of serotonin noradrenaline and dopamine in whole brains of ovariectomised Sprague Dawley rats (250—300 g) Two brains were pooled Number of replicates = 7 The values are means ± standard errors of the mean expressed in μg/g brain

SEROTONIN	0.55 ± 0.02
NORADRENALINE	0.55 ± 0.01
DOPAMINE	0.75 ± 0.03

The analyses of tissue from fraction B 2 yielded rather small amounts of dopamine the fluorescence of the sample taken for estimation was at the lower limit of measurability However, in no case was the read fluorescence less than two times the tissue blank value

Statistical treatment of amine analyses

Controls and experimentals were run at the same time unless otherwise stated Whenever the significance of data was analyzed statistically, Student s t distribution was used

Percentage increases and decreases were calculated as

$$\frac{Expt - control}{control} \times 100$$

The control values were derived from the mean of two estimates of single brains for catecholamines and two for serotonin run at the same time as the experimentals the values are given in the figure legends These controls were spayed and got the same hormone treatment as experimentals taken for amine assay The control animals are not included in the number of animals reported in the text or shown in the figures

Separate controls were run with each experiment because the variability between experiments was much larger than within an experiment

Even if the same treatment of a control group appears in different tables or figures the control experiments were always repeated for each experiment

Results

I The influence of increased cerebral levels of serotonin dopamine and noradrenaline on estrogen progesterone activated estrus behaviour

It has recently been shown that the estrogen progesterone activated heat response is reduced by monoamine oxidase inhibitors (Meyerson 1964 a) The purpose of the present experiments was to study the relationship between this inhibitory effect on the heat response and increased cerebral levels of noradrenaline dopamine and serotonin

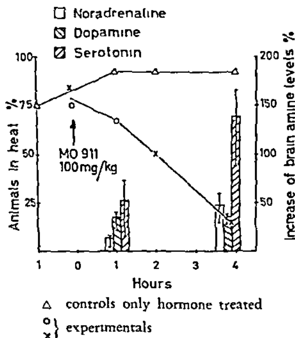


FIGURE 3 The effect of monoamine oxidase inhibition by Mo911 on estradiol progester one activated estrus behaviour and cerebral monoamines in spayed rats
Mo 911 was injected 4 hours after the progesterone administration The curves describe the percentage of animals in heat Each column represents the mean of six amine de terminations on single brains Vertical lines represent range Control values (μg/g) serotonin 0 63 noradrenal ne 0 62 dopamine 0 80 The same control values were used in the 1 and 4 hour experiment The timespan between tests for estrus behaviour and sacri fice for amine analyses was 10—15 minutes

The substance Mo 911 which was used in this experiment has been described as a potent irreversible inhibitor more specific for monoamine oxidase than the hydrazines (Everett, Wiegand and Rinaldi 1963, see also the subsequent discussion by Zeller)

The estrus behaviour was activated by estradiol benzoate, 25 µg/kg followed 48 hours later by progesterone, 0 4 mg/animal

A relationship between the accumulation of cerebral serotonin and noradrenaline and decreased heat response is evident from Fig 3 Only the value of dopamine 1 hour after Mo 911 was almost the same as that at 4 hours, a 40 % increase over the controls

The experiment included 36 animals 12 only hormone treated (controls Fig 3 — △ —), 24 got 100 mg/kg Mo 911 and 12 of these latter were sacrificed for amine analyses 1 hour (Fig 3 — o —) and the other 12 animals 4 hours (Fig 3— x —) after the Mo 911 treatment

The change of the general behaviour of estrogen progesterone treated spayed rats after Mo 911 treatment was described in an earlier investigation (Meyerson 1964 a) The main features 4 hours after injection were that the animals strain when handled and become slightly intractable

Manifestations of heat other than the lordosis reflex such as darting movements and ear wiggling which were seen in most of these animals before the Mo 911 was administered, gradually disappeared and were completely absent at 4 hours after the injection At this time the rejecting kicks with the hind legs when mounted were very frequently seen

In order to study the effect of acute selective increases of dopamine, noradrenaline and serotonin, the corresponding amine precursor DOPA DOPS and 5 HTP were administered 15 minutes after 100 mg/kg Mo 911 (Fig 4) DOPA is a wellknown precursor of dopamine, 5 HTP of serotonin while DOPS is decarboxylated to noradrenaline (see Carlsson 1964)

Tests for heat response were performed one hour after the injection of Mo 911 Fig 4 shows that even greatly increased dopamine and noradrenaline levels did not decrease the heat response, while there was complete absence of estrus behaviour in animals which had recieved 3 mg/kg 5 HTP in combination with the Mo 911 treatment and showed increased serotonin levels

One hour after treatment with Mo 911 in combination with DOPA or 5 HTP the general behaviour was hardly changed Surprisingly the Mo 911 + DOPS treated animals never showed any excitement, but rather decreased activity they sat inactive in their cages but moved about in the observation cage when tested for heat response with normal or slightly reduced exploring behaviour The change of the behaviour seemed not to

be in proportion to the fact that the combination turned out to be toxic 4 out of 10 animals were dead the following day

The estrus behaviour tests were continued with 10 of the Mo 911 + DOPS treated animals Two hours after Mo 911 had been administered 7/10 showed positive heat response and at 4 hours 2/10 These are the same values as obtained by Mo 911 treatment alone (Fig 3) Thus adding DOPS to the Mo 911 treatment neither increased nor decreased the inhibition of the heat response obtained by Mo 911 administration This was true also when DOPA was administered instead of DOPS in similar experiments (Meyerson 1964 a)

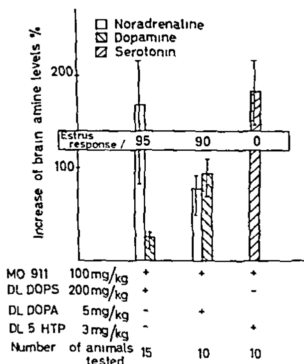


FIGURE 4 The effect of monoamine oxidase inhibition by Mo 911 in combination with the amine precursors DOPS DOPA and 5 HTP on estradiol progesterone activated estrus behaviour in spayed rats Mo 911 was injected 4 hours and the precursors 4 1/4 hours after progesterone administration The estrus behaviour was tested one hour after Mo 911 treatment With these doses 90—95 % of only hormone treated animals responded with heat in control experiments The timespan between test and sacrifice for amine analyses was 10 min Each column represents the mean of five determinations on single brains Vertical lines represents range Control values (μg/g) serotonin 0 51 nor adrenaline 0 45 dopamine 0 78

The substance H 22/54 has been described as a potent catechol O methyl transferase inhibitor, which also blocks the biosynthesis of catecholamines and serotonin, probably by inhibiting the enzymatic hydroxylation of L-phenylalanine, L tyrosine and L tryptophane (Carlsson, Corrodi and Waldeck, 1963) The same authors showed that the increase of cerebral serotonin levels after monoamine oxidase inhibition is prevented by administering H 22/54

In the present experiments H 22/54 was administered half an hour before and one hour after the Mo 911 injection, 2 × 300 mg/kg reduced the accumulation of serotonin as can be seen from Fig 5 compared with Fig 3

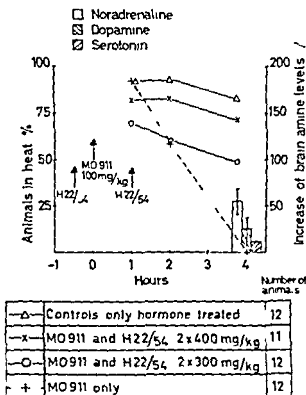


FIGURE 5 The effect of monoamine oxidase inhibition by Mo 911 in combination with the substance H 22/54 2 × 300 mg/kg on estradiol progesterone activated estrus behaviour in spayed rats Mo 911 was injected 4 hours after the progesterone administration The sacrifice for amine analyses was made 4 hours after Mo 911 treatment The curves describe the percentage of animals in heat Each column represents the mean of four determinations on single brains Vertical lines represent range Control values (μg/g) serotonin 0 55 noradrenaline 0 64 dopamine 0 73 The serotonin values − 36 − 36 − 4[illegible] + 71 % change from control

Dopamine and noradrenaline levels were similar to those obtained by Mo 911 alone The inhibitory effect of Mo 911 on estrus behaviour was clearly reduced

A single dose of H 22/54 500 mg/kg 30 minutes before Mo 911 administration in otherwise analogous experiment also tended to protect the estrus behaviour from inhibition by Mo 911 The figures were 8/9 animals responded at 1 hour after Mo 911 treatment and 6/9 at 2 and 4 hours

The change in general behaviour after Mo 911 treatment was not seen in animals which had also been given H 22/54 In contrast to what was seen after Mo 911 alone several animals showed darting movements and ear wiggling when H 22/54 was administered as well However, animals which did not display a positive heat response 4 hours after the Mo 911 administration still displayed the vigorous kicking with the hind legs when mounted indicating that H 22/54 did not prevent these signs of sexual unreceptivity

II Estrus behaviour after estradiol-reserpine treatment

Normally the estrus behaviour is activated by estrogen in combination with progesterone Estrus response after estrogen treatment alone requires very large doses of the hormone (more than 100 µg/kg Meyerson 1964 b) In a recent investigation it was found that reserpine treatment could replace the progesterone administration (Meyerson 1964 b)

The relationship between reserpine-elicited estrus behaviour and the degree of cerebral monoamine depletion after reserpine treatment was studied by taking advantage of the gradual onset of depletion after reserpine

Two dose levels of estradiol were used 2 5 and 10 µg/kg The time from estradiol benzoate injection to estrus behaviour test was kept constant at 56 hours Reserpine 2 mg/kg was injected 1, 3 or 8 hours before the heat response test Ten animals were treated with 10 µg/kg estradiol alone

It is evident from Fig 6, 7 and 8 that less depletion was necessary to obtain a response when the larger dose of estrogen was used In the 10 µg/kg estradiol experiment almost all animals displayed estrus response when about 60 % of the cerebral amines had disappeared Some animals responded already at 10—20 % depletion

A comparison of the extent of noradrenaline, dopamine and serotonin depletion in whole brain after 1, 3 and 8 hours showed that the amines disappeared at very similar rates Therefore these curves do not show which one of the amines is the important one

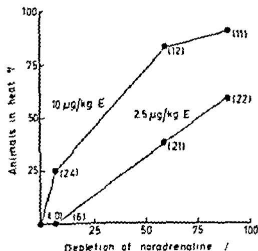


FIGURE 6 The relationship between the estrus response elicited by reserpine 2 mg/kg in estradiol treated (E) spayed rats and the amine levels
Each amine value (abscissa) is the mean of 3—4 estimations on single brains Control values (µg/g) serotonin 0.56 noradrenaline 0.58 dopamine 0.73 The experimentals were taken from the 2.5 µg/kg E experiments The timespan between test and sacrifice for amine analyses was 10 min Numbers within brackets show number of animals tested for estrus behaviour the same animals appear in Figure 7 and 8

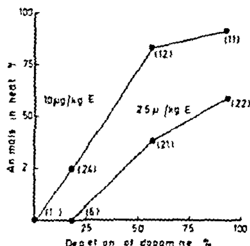


FIGURE 7 For text see Figure 6

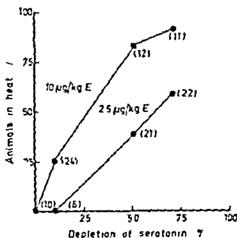
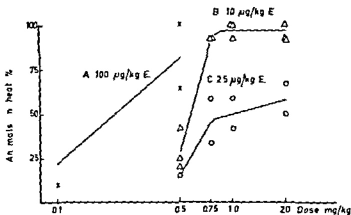


FIGURE 8 For text see Figure 6



Number of animals tested	0.1	0.5	0.75	1.0	2.0		
	22	23				A	x
		34	24	36	32	B	Δ
		12	24	23	22	C	o

FIGURE 9 The relationship between the dose of reserpine and the percentage of estradiol (E) treated spayed rats responding with estrus behaviour
Each point represents one run
Reserpine was injected 48 hours after the administration of E Tests for estrus behaviour were done 8 hours after the reserpine injection

Four dose levels of reserpine were tested after pretreatment 48 hours earlier with 100, 10 or 2.5 μg/kg estradiol benzoate. Fig. 9 shows the heat response 8 hours after the reserpine administration. With the estrogen dose of 100 μg/kg which alone never causes heat response, even 0.1 mg/kg reserpine was sufficient to bring 5 of 22 animals into heat. The data will be further interpreted in the Discussion. Motor activity was only very slightly reduced after 0.5 mg/kg reserpine. Three animals of 23 displayed ear wiggling and 1/23 showed obvious darting movements in the 100 μg/kg estradiol + 0.5 mg/kg reserpine experiment. Such behaviour patterns were observed occasionally even at lower dose levels of estrogen in combination with reserpine 0.5—0.75 mg/kg.

III. The effect of hormone treatment on the cerebral content of noradrenaline, dopamine and serotonin

The cerebral content of noradrenaline, dopamine and serotonin was investigated in spayed rats treated with estradiol benzoate, 50 μg/kg and 48 hours later progesterone 0.4 mg/animal. The animals were sacrificed 5 hours after the progesterone injection. At this time normally 100 % heat response is achieved with the hormone treatment used. Control animals had oil blank solutions instead of hormones.

The diencephalic-mesencephalic parts were dissected out according to method A for serotonin analyses and method B for catecholamine analyses. Method A was easier to repeat consistently due to the fact, that blunt dissection was possible to a great extent. However, method B was chosen for

TABLE 2
The effect of estradiol progesterone treatment on the cerebral content of serotonin in spayed rats.
Progesterone 0.4 mg/animal was injected 48 hours after estradiol benzoate 50 μg/kg. The animals were sacrificed 5 hours after the progesterone injection. Each replicate (5) consists of tissue from 5 brains. The values are means ± standard errors of the mean expressed in μg/g brain.

DISSECTION METHOD A	Hormone treated	Controls
Fraction 1	0.84 ± 0.05[1]	0.72 ± 0.04[1]
Fraction 2	0.48 ± 0.01	0.44 ± 0.02

[1] P of difference > 0.1

TABLE 3

The effect of estradiol progesterone treatment on the cerebral content of noradrenaline (NA) and dopamine (DA) in spayed rats Progesterone 0 4 mg/animal was injected 48 hours after estradiol benzoate 50 μg/kg The animals were sacrificed 5 hours after the progesterone injection Each replicate (4) consists of tissue from 5 pooled brains The values are means ± standard errors of the mean expressed in μg/g brain

DISSECTION METHOD B		Hormone treated	Controls
Fraction 1	NA	2 09 ± 0.22[1]	2 18 ± 0 2[illegible][1]
	DA	0 80 ± 0 16	0 97 ± 0 17
Fraction 2	NA	0 93 ± 0 18	0 98 ± 0 17
	DA	0 27 ± 0 05	0 33 ± 0 08
Fraction 3	NA	0 40 ± 0 05	0 45 ± 0 02
	DA	1 07 ± 0 17[2]	1.24 ± 0 06[2]
Fraction (1 + 2 + 3)	NA	0 55 ± 0 04	0 61 ± 0 004
	DA	0 98 ± 0 15[3]	1 13 ± 0 07[3]

P of difference [1] > 0.5 > 0 2 [3] > 0.2

the catecholamine analyses as the dopamine rich regions nucleus caudatus and nucleus lentiformis were more completely separated by this method from the diencephalic part

Noradrenaline in the hypothalamus has been shown to be concentrated in areas mainly within the anterior parts (Carlsson Falk and Hillarp 1962) The amount of noradrenaline in the present investigation is in good agreement with that obtained by the same authors in the posterior and anterior intermediate hypothalamus (1 2 resp 1 9—2 3 μg/g)

It is evident from Table 2 and 3 that there is no statistically significant difference in the amount of assayed monoamines in the control and the combined hormone treated animals

Nor are there statistically significant effects on the cerebral amines in opposite direction by estrogen and progesterone (Table 4 5)

Good agreement between analyses of the two parts of divided homogenates and satisfactory recoveries make it probable that the rather large scatter of some of the catecholamine results was due to the difficulty in re-

TABLE 4

The effect of progesterone or estradiol treatment on the cerebral content of serotonin in spayed rats. The animals were sacrificed 5 hours after 4 mg/animal progesterone resp. 53 hours after 50 µg/kg estradiol benzoate. Each replicate (3) consists of tissue from 5 brains. The values are means ± standard errors of the mean expressed in µg/g brain.

DISSECTION METHOD A	Progesterone treated	Estradiol treated
Fraction 1	0.78 ± 0.02	0.79 ± 0.04
Fraction 2	0.50 ± 0.03	0.47 ± 0.01

The two groups of treatment were not run at the same time.

TABLE 5

The effect of progesterone or estradiol benzoate treatment on the cerebral content of noradrenaline (NA) and dopamine (DA) in spayed rats.
The animals were sacrificed 5 hours after 4 mg/animal progesterone resp. 53 hours after 50 µg/kg estradiol benzoate. Each replicate (3) consists of tissue from 5 brains.
The values are means ± standard errors of the mean expressed in µg/g brain.

DISSECTION METHOD B		Progesterone treated	Estradiol treated
Fraction 1	NA	1.95 ± 0.05	1.97 ± 0.06
	DA	0.91 ± 0.10[1]	1.20 ± 0.12[1]
Fraction 2	NA	1.03 ± 0.05	1.05 ± 0.11
	DA	0.48 ± 0.05	0.37 ± 0.06
Fraction 3	NA	0.41 ± 0.05	0.42 ± 0.03
	DA	1.00 ± 0.06	1.00 ± 0.07
Fraction (1 + 2 + 3)	NA	0.56 ± 0.04	0.59 ± 0.05
	DA	0.95 ± 0.06	0.95 ± 0.03

[1]P of difference > 0.1. The two groups of treatment were not run at the same time.
Test of significance of the difference between DA fraction 1 Table 5 estradiol treated and DA fraction 1 Table 3 hormone treated P > 0.1

producing the dissections exactly from experiment to experiment. As controls and experimentals were always run at the same time (except in Table 4 and 5) this possible inconsistency affects both treated and not hormone treated alike.

TABLE 6

The cerebral serotonin content in spayed rats after treatment with estradiol benzoate 50 μg/kg s c followed by progesterone 0 4 mg/animal s c 48 hours later and Mo 911 50 mg/kg i v., at 52 hours The animals were sacrified one hour after Mo 911 treatment Each replicate (5) consists of tissue from 3 brains The values are means ± standard errors of the mean expressed in μg/g brain

DISSECTION METHOD A	Hormone treated	Controls
Fraction 1	1 31 ± 0 06	1.35 ± 0 04
Fraction 2	0 79 ± 0 05	0 79 ± 0 04

An attempt was made to determine a possible effect on the turnover of brain serotonin by estrogen progesterone treatment The monoamine oxidase inhibitor Mo 911 was injected 50 mg/kg intravenously and the animals were sacrified one hour later At this time the decreased monoamine breakdown had resulted in an accumulation of serotonin of about 60—70 % (Compare Table 2 and 6) This response is within submaximal limits The serotonin increase was the same whether the hormones had been administered or not (Table 6)

Discussion

The effect of increased cerebral monoamine levels

The recently described inhibitory effect on the estradiol progesterone activated estrus behaviour by monoamine oxidase inhibitors (Meyerson 1964 a) has been given added weight by the results of the present investigation with the monoamine oxidase inhibitor Mo 911 The investigation shows that the decreased heat response is very likely due to the increase of cerebral serotonin levels

The relationship between decreased heat response after Mo 911 treatment and increasing levels of serotonin and noradrenaline is evident (Fig 3) The dopamine level was however not changed during the increasing heat inhibitory phase which could be seen by comparison of the 1 hour and 4 hour values in Fig 3

A comparison of the serotonin, dopamine and noradrenaline levels at 4 hours after Mo 911 treatment, i e when heat inhibition is very obvious with the more selective increase of the amines after Mo 911 in combination with the amine precursors indicate that it is the serotonin increase which interferes rather than that of dopamine or noradrenaline (fig 4) This seems true even if a delay in the release to the effector cell of freshly accumulated catecholamines could possibly exist when the increase is caused by means of exogenous precursors Such a delay is suggested by the fact that the animals general behaviour was very little changed at 1 hour after Mo 911 + precursor treatment (see also Meyerson 1964 a Table VII) in spite of high amine levels However it has been shown in earlier work (Meyerson 1964 a) as well as in the present investigation that the heat inhibition observed 4—6 hours after Mo 911 is not increased if DOPA or DOPS are administered with the Mo 911 At such a long time after the injection the selectively increased catecholamines should be available at the receptor sites Thus there was no demonstrable effect on the heat response of acutely increased or more prolonged increased availability of catecholamines

It might be said that an inhibitory effect on the heat response of catecholamines could not be fully excluded by merely correlating the heat inhibition with the brain amine levels (Fig 3) For instance the unaltered dopamine level during the increasing heat inhibitory phase after Mo 911 may be due to an increasing release to the effector cells and subsequent degradation by catechol O methyl transferase (COMT)

The presence of COMT in the brain was demonstrated by Axelrod (1958) Accumulating evidence supports its physiological significance The 3 O methylated catecholamines normetanephrine and 3 methoxytyramine, occur normally in the brain as was shown by Haggendahl (1963) and dis appeared after treatment with COMT inhibiting substances The possibility that the monoamine oxidase enzyme is close to the site of synthesis and that COMT occurs extracellularly near the effector cell was discussed by Carlsson (1960, 1964) It is suggested by the rapid accumulation of cerebral noradrenaline after administration of monoamine oxidase inhibitor in con trast to the rise in normethanephrine which follows some hours later (Carlsson, Lindqvist and Magnusson 1960) Similarly, when L-DOPA is injected into rabbits intravenously 3 4 dihydroxyphenyl acetic acid is ac cumulated and only later homovanillic acid (Carlsson and Hillarp 1962)

Alpha substituted dopacetamid derivatives have recently been described as potent COMT inhibitors (Carlsson, Corrodi and Waldeck 1963) The in- hibitory effect on the heat response of Mo 911 was decreased when this treatment was combined with the COMT inhibitor H 22/54 This substance should bring about increased availability of catecholamines at postsynaptic receptors The simultaneous decrease in catecholamine synthesis by phenyl alanine and tyrosine hydroxylase inhibition seem to be of less importance The dopamine and noradrenaline levels were nearly the same after Mo 911 + H 22/54 as after Mo 911 alone Thus the results indicate that the reduced estrus response after Mo 911 is not due to decreased degradation of *catecholamines* If an increase in catecholamines were heat inhibitory H 22/54 should not have counteracted Mo 911

The decreased effect on the heat response of Mo 911 when combined with H 22/54 is probably due to inhibition of the *serotonin* synthesis by the inhibitory effect of H 22/54 on tryptophane hydroxylase (Carlsson Corrodi and Waldeck 1963)

The effect of amine depletion

In a previous paper the possibility was discussed that amine depletors (reserpine tetrabenazine) removed monoaminergic heat inhibition (Meyer son 1964 b)

The fact that estrus behaviour was not activated in the absence of estro gen administration might indicate that exciting activity mediating estrus behaviour does not exist continuously but only after the estrogenic hormone has been in action

The present investigation shows that if the dose of estrogen is increased less amine depletion is required to elicit the estrus response (Fig 6, 7 and 8) The more excitation, the less heat inhibition needs to be removed

The fact that a low degree of depletion is sufficient to elicit the heat response indicates that either the relevant structures have to be only partially depleted of transmitter or that they constitute a part of the brain especially sensitive to reserpine

The fact that the effect of a single dose lasts for several weeks indicates that the relevant structures recover very slowly As shown by Carlsson Falck and Hillarp (1962) and Dahlstrom and Fuxe (1964) using a histochemical fluorescence method for demonstration of monoamines, the terminals are more easily depleted than the cellbodies and recover more slowly The data from the work of Carlsson et al concern catecholamine terminals However the said is also true for serotonin terminals (Fuxe, 1964) Within the suprachiasmatic nucleus the serotoninergic fibers stands out very distinctly The yellow fluorescence characteristic for serotonin was very much reduced within this region even 10 days after 2 mg/kg reserpine in ovariectomised rats treated in addition to the reserpine treatment with 25 μg/kg estradiol 36 hours before they were sacrificed (Dahlstrom, Fuxe and Meyerson unpublished data) It seems reasonable therefore to conclude that the observed removal of heat inhibition is due to the effect on terminals

The effect of hormone treatment on brain amine content

It could be concluded from the results of the present investigation that an estradiol − progesterone treatment which normally activates estrus behaviour did not bring about changes of cerebral serotonin dopamine or noradrenaline levels compared with untreated controls Nor were there any statistically significant changes in the amine levels when the hormones were given separately

However the results do of course not preclude the possibility of highly *localised quantitative changes not detected by the method used*

Estrus behaviour and monoamines

Summary and Conclusions

[The following summary is based upon the present (III) and two earlier papers Meyerson, 1964 a (I) and 1964 b (II)]

If the present and earlier data are taken together there is good evidence for the assumption that in the female rat there exist monoaminergic pathways which mediate heat inhibition

An increase in monoaminergic tone would activate heat inhibition while a decrease would remove such an inhibition

A A general increase of the monoamine levels in the brain by different monoamine oxidase inhibitors (Mo 911 Nialamide JB 516) brought about decreased estrogen progesterone activated heat response (I) A more selective accumulation of serotonin dopamine or noradrenaline achieved by precursor treatment revealed an obvious inhibition by increased serotonin levels while the catecholamine increase had no apparent effect (I and III)

Supporting the view that the effect of the monoamine oxidase inhibitors is due to the increased levels of serotonin is the fact that the effect of Mo 911 was less when combined with the tryptophane hydroxylase inhibitor H 22/54 (III)

Even if there is good evidence that increased levels of serotonin bring about the heat inhibition the existence of special serotoninergic heat inhibitory receptors is still uncertain

Serotonin might conceivably act at dopaminergic or noradrenergic receptors with competitive blocking of a heat stimulatory response

Against this possibility speak the following findings Heat inhibition at 2 or 4 hours after injection of Mo 911 alone was almost the same as in the experiments where DOPS (III) or DOPA (I) was injected as well In these later cases greatly increased amounts of noradrenaline and dopamine were available

Nor seems monoaminergic heat stimulation likely in regard of the effect of reserpine on estrus behaviour (see below)

B Progesterone could be replaced by amine depletion caused by reserpine or tetrabenazine (II)

The effect of reserpine was not prevented by adrenalectomy This excludes the possibility that is was caused by the secretion of progestative substances from the adrenals due to reserpine released ACTH (II)

The results from dose response experiments indicate that the effect of reserpine might be at the terminal part of the axon (III), a response was elicited by a dose level reportedly insufficient to give a demonstrable effect on the nerve cell bodies and a response was achieved a long time after a single dose of reserpine (II) indicating slow recovery which is more characteristic for the nerve terminals than the nerve cell bodies (cf Carlsson et al 1962 Dahlstrom and Fuxe 1964)

It must be pointed out, that the discussion and conclusions above are partly based upon analyses of the amine content of extensive regions of the brain This might be risky in view of the accumulating evidence that amines are stored within the neuron in various pools with different functional significance (see Kopin 1964) and also considering the possibility of highly localized changes within certain areas or nuclei

C The inhibition of the estrogen progesterone activated heat response was very evident after α methylDOPA administration (I) The effect of α methylDOPA was shown very probably to be due to decarboxylated metabolites as the effect was reduced by pretreatment with decarboxylase inhibitors Considering the heat facilitating effect of reserpine (II) the improved heat inhibition of α methylDOPA in animals pretreated with reserpine (I) the absence of inhibitory effects of decarboxylase inhibitors (I) and of hydroxylase-COMT inhibitors (H 22/54 unpublished observation), it seems possible to conclude that the effect of α methylDOPA is not due to amine synthesis inhibition or displacement of endogenous amines A direct effect of α methylDOPA metabolites on heat inhibitory receptors must be considered

DL amphetamine reduced the estrogen progesterone activated heat response (I) Vane (1960) demonstrated that D amphetamine acts on tryptamine receptors in the rat stomach and Innes (1963) could extend this conclusions to guinea pig ileum dog retractor penis rabbit aorta and rabbit uterus It therefore seems possible that amphetamine and perhaps also α methylDOPA metabolites are able to stimulate central nervous serotoninergic heat inhibitory receptors

As an indicator that the animals are in heat the lordosis response stimulated by mounting has been used throughout the investigation Also other manifestations of estrus behaviour were activated by reserpine in estrogen pretreated spayed rats (III) It therefore seems probable that the monoaminergic heat inhibition includes other manifestations of heat in addition to the lordosis response

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THE EFFECT OF DIFFERENT PROSTAGLANDINS ON HUMAN MYOMETRIUM IN VITRO

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INTRODUCTION

In the beginning of the 1930 s it was found that human seminal fluid contains a factor with smooth muscle stimulating properties (Euler 1934, Goldblatt 1933 1935) Euler (1935) named it provisionally Prostaglan din , and this term will be used, for simplicity, for the different crude extracts used in earlier studies

Purification and chemical elucidation

The purification of prostaglandin was started by Euler (1935, 1936, 1939), *who showed that prostaglandin is a weak organic carboxylic acid, probably* unsaturated, and containing hydroxy groups

Euler defined prostaglandin as the lipid, soluble smooth muscle stimulating and blood pressure lowering factor with acidic properties found in seminal fluid and extracts of some accessory genital glands of man and sheep When testing prostaglandin for its biological properties Euler (1939) used a barium salt preparation 0 1 mg of which corresponded to 1 unit The same preparation as used by Euler was also employed by Asplund (1947 a) in his experiments Eliasson s purified prostaglandin had the same activity expressed in units as Euler s extract, when bio assayed on isolated rabbit jejunum (Eliasson 1959)

The purification work and the elucidation of the chemical structure has been continued by Bergstrom and co workers in recent years Starting with the above mentioned barium salt and extract of vesicular glands from sheep, Bergstrom and Sjovall (1960 a, b) were able to crystallize two compounds named PGE and PGF with the chemical formulas $C_{20}H_{34}O_5$ and $C_{20}H_{36}O_5$ respectively It was later shown that the vesicular glands also contain other compounds with similar biological properties and the first crystallized compounds were therefore renamed PGE_1 and $PGF_{1\alpha}$ Their chemical structure was clarified by Bergstrom *et al* (1962 d) In further investigations the presence of two additioned compounds called prostaglandin E_2 and E_3 in vesicular glands from sheep was determined Their chemical structure has been established by Bergstrom *et al* (1962 b) and Samuelsson (1963 a) Their chemical formulas are given in Fig 1 As shown in Fig 1, all PG compounds have a similar chemical structure

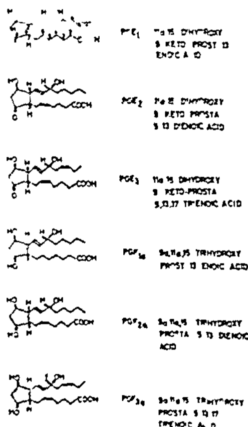


Fig 1 Chemical formulas for the prostaglandins

While the PGE have a keto group at C 9 the PGF have a hydroxy group in this position The PGE compounds differ from each other by their number of double bonds From the PGE six different PGF can be formed by reduction of the keto group resulting in the formation of two stereoisomers from each of PGF_1, PGF_2 and PGF_3 (Bergstrom *et al* 1962 b, c) These were designated $PGF_{1\alpha}$ and β etc

Distribution

Euler (1934 1935) and Goldblatt (1933 1935) found prostaglandin in seminal fluid from man The content was 20—60 units per ml in apparently fertile men (Eliasson 1959) Semen from patients submitting samples for analysis because of sterility contained however only 8—12 units (Asplund 1947 a Eliasson 1959) The pure PG compounds present in human semen are PGE_1 PGE_2 PGE_3 $PGF_{1\alpha}$ and $PGF_{2\alpha}$ (Bergstrom and Samuelsson 1962 Samuelsson 1963 b) Samuelsson (1963 b) calculated

the content of these compounds through their biological activity to 20 μg of PGE_1, PGE_2 and PGE_3 and to 3—5 μg of $PGF_{1\alpha}$ and $PGF_{2\alpha}$ per ml semen, obtained from sterility clinics Recent chemical determinations on single samples of semen give almost the same values although the content of E_3 was lower (Bygdeman and Samuelsson 1964) The prostaglandins are also present in accessory genital organs as vesicular gland, prostate gland, ampulla ductus deferens of sheep and human seminal vesicles (Euler 1934, 1935, 1936, Goldblatt 1933 1935, Eliasson 1959) The pure PG-compounds found in sheep vesicular gland are PGE_1, PGE_2, PGE_3 and $PGF_{1\alpha}$ (Bergstrom and Sjovall 1960 a b, Bergstrom *et al* 1962 b) Eliasson (1959) estimated the concentration of prostaglandin in sheep accessory genital organs to 2 0—5 0, 2 0—2 4 and 1 5—2 0 units per gram wet weight for the vesicular gland the ampulla ductus deferens and the prostate gland respectively

Eliasson (1959) investigated where prostaglandin in semen is formed By using human split-ejaculates he found a correlation between the content of fructose and prostaglandin in the different portions of the ejaculates, indicating that prostaglandin is formed in the seminal vesicles If the different accessory genital glands from sheep were separately in cubated in phosphate buffer the yield of prostaglandin increased in the vesicular gland and in the ampulla ductus deferens from 2 0—5 0 to 40—80 and from 2 0—2 4 to 30—40 units per g respectively The content in the prostate and Cowper s gland was unchanged Recently also direct evidence has been published showing the ability of vesicular glands from sheep to synthetizise different PGE compounds *in vitro* If y homolinolenic acid, arachidonic acid or an eicosa pentaenoic acid was incubated at 37°C with homogenized vesicular glands from sheep, PGE_1, PGE_2 or PGE_3, respectively, was formed The yield was calculated to about 20% (Berg strom *et al* 1964 a b, Dorp *et al* 1964 a, b)

Many other types of semen such as from bull, boar, dog rabbit, pig and horse have been investigated with negative results with regard to their content of prostaglandin (Euler 1934 1936, Eliasson 1959) Goat seminal fluid, however, contains about 12 units of prostaglandin per ml (Eliasson 1959) Semen from monkey also has smooth muscle stimulating activity which is due to its content of a substance, vesiglandin , similar to prosta glandin (Euler 1935 1936)

Even different other organs have been investigated Thus Euler and Hammarstrom (1937) found less than 1% of the activity present in sheep vesicular gland tested on rabbit isolated jejunum in blood, brain and pancreas of sheep Eliasson (1959) found minor quantities of lipid

soluble smooth muscle stimulating activity in sheep's lung, kidney and liver. The activity differed from that found in the vesicular glands as it had no effect on blood pressure. During recent years many organs have been examined with regard to their content of pure PG compounds. Due to the new methods developed small quantities have also been detected. Thus sheep lungs contain PGE_2, $PGF_{2\alpha}$ and PGF_2 (Bergstrom *et al.* 1962 a, Änggard and Samuelsson 1963). $PGF_{2\alpha}$ is also present in lungs from pig, man, monkey and guinea pig (Bergstrom *et al.* 1962 a, Änggard 1964). Approximate concentrations for $PGF_{2\alpha}$ per gram wet weight calculated by means of isotope dilution were: human lung 0.02 μg, monkey lung 0.2 μg and guinea pig lung 0.5 μg (Änggard 1964). The pure PG compounds have also been detected in thymus (Bergstrom and Samuelsson 1963), iris (Änggard and Samuelsson 1964) and human menstrual fluid (Eglington *et al.* 1963).

Biological effect

The review of the biological effects of the prostaglandins is divided in two parts, the first including the effect on different smooth muscle and other organ systems and the second concerning the effect on human myometrium.

a) *Effect on different organs*

The effect of prostaglandin and the different pure PG compounds, PGE_1, PGE_2, PGE_3, $PGF_{1\alpha}$ and $PGF_{2\alpha}$ on smooth muscle is in most cases qualitatively the same. Normally the prostaglandins stimulate almost all the smooth muscle preparations tested, as isolated jejunum or duodenum from rabbit, guinea pig, rat, mouse, cat, hamster, myometrial preparations from rabbit, guinea pig and rat (Euler 1936, 1939, Eliasson 1959, Bergstrom *et al.* 1959 b, Änggard and Bergstrom 1963, Bergstrom and Euler 1963, Horton and Main 1963). The threshold values *of the different* substances vary considerably. If for instance PGE_1 and $PGF_{1\alpha}$ are compared, the sensitivity of guinea pig ileum to $PGF_{1\alpha}$ is 5 to 10 times higher than to PGE_1. The opposite condition is found on stilbol treated rabbit uterus (Bergstrom *et al.* 1959 a). $PGF_{2\alpha}$ is normally slightly more active than $PGF_{1\alpha}$ (Änggard and Bergstrom 1963). On rabbit jejunum, which seems to be one of the most sensitive organs, the threshold values for $PGF_{1\alpha}$ and $PGF_{2\alpha}$ are 0.001–0.003 μg per ml bath fluid. Among the three PGE-compounds PGE_1 usually is the most effective one (Bergstrom and Euler 1963, Horton and Main 1963).

Prostaglandin and the PG compounds lower the blood pressure. This effect of prostaglandin has been tested on rabbit, cat and dog (Euler 1936 and 1939, Eliasson 1959), of E_1 on rabbit and human (Bergstrom and Euler 1963, Horton and Main 1963, Bergstrom *et al* 1959 a, b), and of E_2 and E_3 on rabbit (Bergstrom and Euler 1963, Horton and Main 1963). One unit of prostaglandin depresses the blood pressure of rabbit with 30%. The threshold dose of PGE_1 in this respect is 0.5 μg per kg on the same animal. The effect of PGE_2 is the same or slightly more pronounced and of PGE_3 definitely lower than that of PGE_1. In humans 0.2—0.7 μg PGE_1/kg/min gave a fall of both the systolic and diastolic blood pressure, a small decrease in the cardiac output although the pulse rate was increased (Bergstrom *et al* 1959 a). $PGF_{1\alpha}$ and $PGF_{2\alpha}$ also decrease the blood pressure of rabbit and cat although the dose needed is about 15—20 times higher than for PGE_1 (Horton and Main 1963, Bergstrom *et al* 1959 b, Änggard and Bergstrom 1963). An increased bronchial resistance was following i.v. injection of $PGF_{2\alpha}$ in cat, an effect which could be due to constriction of the bronchial smooth muscles and is of interest with regard to the fact that $PGF_{2\alpha}$ normally is present in this organ (Änggard and Bergstrom 1963).

The Fallopian tubes of rabbit and human are also exceptions from the general rule that the prostaglandins stimulate smooth muscles. The PGE compounds and prostaglandin thus decreased the tone of this organ both *in vivo* and *in vitro* (Asplund 1947 b, Horton and Main 1963, Horton, Main and Thompson 1963). $PGF_{1\alpha}$ showed, however, the usual stimulatory effect (Horton and Main 1963). Sandberg, Ingelman-Sundberg and Rydén (1963 a, b), and Sandberg *et al* (1962) described an inhibitory action of both prostaglandin and PGE_1 on human Fallopian tube with the exception for the most proximal part which was contracted.

Even in other quite different fields effects of some of the PG compounds have been published. All the PGE compounds for instance caused a significant fall in the free fatty acids (FFA) in the blood and PGE_1 almost completely blocked the usual increase of FFA caused by adrenaline (Bergstrom, Carlsson and Oro 1964). Another example is their effect on the central nervous system. Injected into the cerebral ventricles of unanaesthetized rats the PGE compounds produced sedation, stupor and signs of catatonia (Horton 1964).

b) *Effect on human myometrium*

That human seminal fluid could affect the motility of human myometrium was first described in the beginning of the 1930's by Kurzrok and Lieb

(1930) and by Cockrill Miller and Kurzrok (1935) They used in their experiments isolated strips obtained from uteri usually excised because of *myoma* The strips were mounted in organ baths, and the effect of single semen samples was tested The material consisted of about 400 uterine strips Normally the semen caused an inhibition of the spontaneous motility 10 per cent of the strips reacted, however, with stimulation for all semen samples tested In a later investigation a correlation was found between these uteri and a long lasting primary or secondary sterility in the corresponding women

In vitro prostaglandin normally inhibited the spontaneous motility of human myometrium At ovulation time the threshold dose was 0 002—0 01 units per ml bath fluid Myometrial pieces from women in menopause or with hormonal disturbances such as *metropathia hemorrhagica cystica* seemed to be less sensitive to prostaglandin In this material, including eight recorded strips, five were in late proliferative phase Two of these five reacted with a slight stimulation characterized by an increased amplitude of the contractions and a tendency of synchronizing the activity to a small dose of prostaglandin When the dose was increased the normal inhibition was obtained (Eliasson 1959)

The sensitivity of the myometrium to either human semen or prostaglandin in experiments *in vivo* as well as *in vitro* seems to vary with the phase in menstrual cycle Karlson (1949, 1959), and Eliasson and Posse (1960) have studied this problem using the same method with three separate pressure transducers as described by Karlson (1944) In his experiments Karlson tested the effect of the husband s semen instilled in the vagina of infertile women In the tests made at ovulation time normally after 2 3 min an increased motility in the corpus part was found, while the activity in cervix and isthmus was decreased The effect lasted for 10 20 min However in some cases the semen caused a relaxation of the whole organ which Karlson thought to be abnormal and the cause of infertility During the secretory phase usually all parts of the uterus were stimulated

In contrary to Karlson s experiments Eliasson and Posse made their test on young fertile women with one or several children They instilled 7[illegible] 1[illegible] units of prostaglandin in the vagina a dose which corresponds well to the amount found in a normal ejaculate During the proliferative and secretory phases with exception of the time around ovulation as well as during menstruation prostaglandin in these doses had no effect At ovulation time however prostaglandin initially increased the activity but the effect changed to inhibition after 20—25 min Of interest was the

observation that if the sensitivity of the myometrium was increased by a simultaneous intravenous infusion of post pituitary hormones (Csapo 1954), prostaglandin caused an obvious inhibition of the motility after a few minutes A decrease of the motility of the myometrium *in vivo* after coitus has been demonstrated by Bickers and Main (1941) using the balloon technique

Physiological role of the prostaglandins

Previously at least two physiological functions of prostaglandin in human reproduction have been suggested Euler (1936) assumed that the strong smooth muscle stimulating effect of prostaglandin might be of importance for the emptying of the male accessory genital glands

The proposed physiological role of prostaglandin in female reproduction has been the subject of several studies, and as it is of particular interest for the present study, it will be described here more in detail Bickers found (1951) that hypertonic arrhythmic contractions of the myometrium markedly decreased the sperm migration Normally prostaglandin and human semen inhibited the spontaneous motility of human myometrium (Kurzrok and Lieb 1930 Cockrill *et al* 1935, Eliasson 1959, Eliasson and Posse 1960) However an abnormal stimulatory effect of human semen was found in some cases and could be correlated to a prolonged infertility (Cockrill *et al* 1935) It is possible that the stimulatory effect of semen in these cases might have caused an abnormal motility *in vivo* corresponding to that described by Bickers (1951) and consequently a delayed transport of the spermatozoa Due to these and other results Eliasson (1959) proposed the hypothesis that the physiological role of prostaglandin is to facilitate a passive sperm transport Experiments indicating such a transport *in vivo* in human has been presented by many authors (c f Hartman 1957, Belonoschkin 1949 1959) Other investigators however, have not been able to confirm these results The marked decrease in tonus normally evoked by prostaglandin 2 to 5 min after ejaculation seems likely to give a suction effect which transports the sperms from semen pool around the portio into the uterine cavity

Also the effect of the prostaglandins on the Fallopian tube might be of importance for conception (Horton and Main 1963 Sandberg *et al* 1963 b)

The distribution of the prostaglandin compounds in many organs and their effect on different smooth muscle systems as well as on fat metabolism suggests a more wide function than initially presumed

Present study

Earlier studies indicate that seminal fluid and prostaglandin influence the spontaneous motility of human myometrium both *in vivo* and *in vitro*. In these studies single human semen samples with unspecified smooth muscle activity (with a few exceptions) were used. It therefore seemed to be of interest to investigate the effect of a total extract of human semen, HSF PG, on a greater number of human uteri and to compare its effect with that of the pure PG-compounds PGE$_1$, PGE$_2$, PGE$_3$, PGF$_{1\alpha}$ and PGF$_{2\alpha}$ present in human semen. As the amounts of each of these compounds in HSF PG now could be estimated, the importance of the individual fractions constituting the effect of HSF PG could be examined. This is of interest since the proportion and amount of PG-compounds may show wide variations in single semen samples (Bygdeman and Samuelsson to be published).

The sensitivity and reactivity pattern of human myometrium was also investigated under different hormonal conditions. An increased sensitivity of the myometrium, especially at the time of ovulation, would further advocate the proposed role of prostaglandin in conception.

The mode of action of prostaglandin on human myometrium is quite unknown. This problem was investigated with regard to factors as ions and hormones, which are known to be of importance for the reactivity pattern and sensitivity of other myometrial preparations. The investigation was performed *in vitro*.

Reports of some of the results included in the present study have been published previously (Bygdeman and Eliasson 1963 a, b and c).

CHAPTER II

METHODS

Registration technique

As test material human myometrium was used. The myometrium has been obtained from patients undergoing hysterectomy due to myomatous changes in the uterus and from pregnant uteri in the 12th to 20th week of gestation when legal abortion by abdominal evacuation in connection with sterilization was made. In a few cases even pregnant myometrium at term was used. Immediately following the surgical removal of the uterus or when the pregnant uterus was opened, suitable pieces of apparently normal myometrium were taken longitudinally from the corpus part and suspended in cold Ringer's solution. Four to eight strips of equal size (about 20×2×2 mm) were then cut out, each mounted in a separate 40 ml cuvette in an isolated organ bath. This could be done within one hour. In all cases except the pregnant ones, part of the uteri were sent to histological examination for determination of the phase in the menstrual cycle and the diagnosis. The salt solution used was Tyrode solution modified according to Genell (1937) and aerated with 5 per cent CO_2 in O_2. The gas flow was adjusted to give a pH of 7.35 ± 0.05 and controlled continuously or at 15 min intervals by a pH meter (radiometer PHM22 electrode GK 2024C) connected to an ink writer. The bath fluid passed continuously from below at a constant rate of 1.5 ml per minute controlled by drop chambers. The bath temperature was kept at 37.5°C by a temperature controlled water bath. The motility of the strips was recorded almost isometrically using frontal writing levers on a smoked drum, with an amplification of 25:1. The writers were loaded with known weights until an amplitude of the recorded spontaneous motility of about 2—4 cm was reached. This technique involves activation of all the muscle cells in the myometrial strip during the contraction (Csapo 1954).

By using a continuous flow of Tyrode solution washing in the usual way was avoided. This fact is of importance since the spontaneous motility of human myometrium is disturbed by washing in the usual way, apparently because of its sensitivity to even slight variations in pH (Mark 1961), and to changes in stretch caused by the sudden variation in weight. The continuous flow is also of advantage when for instance the ion content of the bath should be changed. When the variation is made in the reservoir

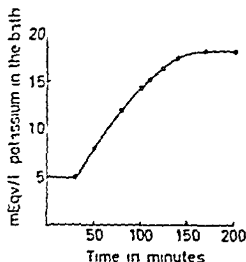


Fig 2 Potassium concentration in the organ bath when the concentration in the reservoir bottle has been changed (at zero time) After 1½ hour the potassium content of the bath is almost the same as in the reservoir

bottles the concentration of the substance in the bath is slowly changed until equilibrium is reached Sudden changes are then avoided (Fig 2) The disadvantage of this procedure is, however, that substances added directly to the bath are only slowly washed out. The time course was investigated by adding red colour to the bath The concentration after different time intervals was estimated on a Beckman spectrophotometer (Model B) at 435 mμ As illustrated in Fig 3 the half time of the concentration in the bath with the used flow of Tyrode solution was about 20 min After 1½ hour almost all of the added substance was washed away Therefore as a rule substances were added to the bath not more often than every 1½ hour

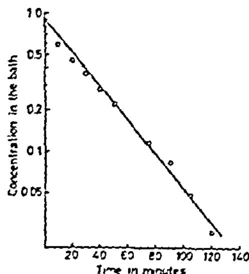


Fig 3 The time concentration relationship [illegible] added to the bath Half time [illegible] After 1½ hour more than [illegible] of the substance is washed away

The prostaglandins tested in this work were PGE_1, PGE_2, PGE_3, $PGF_{1\alpha}$, $PGF_{2\alpha}$ and a total extract of human semen, HSF PG The pure PG compounds were kindly put to my disposal by Prof S Bergstrom and Dr B Samuelsson The substances were added to the organ baths and their effect on the spontaneous motility examined The experiments started normally 2—3 hours after the myometrial strips were mounted At this time the spontaneous motility had reached a steady state HSF PG was extracted from a large pool of human semen (250—500 ml) according to Eliasson (1959) The semen was obtained from sterility clinics and consisted of samples delivered for investigation They were fetched within an hour after the delivery and then kept at —20°C until the extraction By using a large pool of semen individual variations in the samples with regard to their content of the different prostaglandin compounds could be avoided

When the experiments were started in 1961 no method was available for estimations of the content of the different prostaglandin compounds in an extract from human semen Therefore the activity of HSF PG was expressed in units according to its activity on isolated rabbit jejunum and rabbit blood pressure (Euler 1936, 1939, Eliasson 1959) The effect of the extract was compared with a standard preparation of prostaglandin put to my disposal by Dr Eliasson Recently such a method has been presented (Bygdeman and Samuelsson 1964) The method includes group separation on silicic acid column into PGE and PGF compounds The PGE are further separated by thin layer chromatography and determined by treatment of the eluates with KOH and measurement of the developing ultraviolet absorption at 278 mμ The PGF compounds are separated and determined after conversion into methylesters and trimethyl silyl ether derivatives by gas liquid chromatography The results are given in Table 1 Three estimations were made both on the initial pool of semen and on the HSF PG extract from the pool HSF PG apparently contains all the PG compounds present in human semen although the recovery of PGE_2 and PGE_3 was slightly less than that found for the other compounds The biological effect of the extract corresponded to 6 units per ml semen tested on rabbit isolated jejunum and rabbit blood pressure The HSF PG extract was also assayed on rabbit jejunum and rabbit blood pressure in comparison with E_1 and E_2 With corrections for the known content of E_3 $F_{1\alpha}$ and $F_{2\alpha}$ the content of PGE_1 and PGE_2 was 12 and 8 μg respectively

If the content of the different prostaglandins found in HSF PG is expressed as PGE_1 units by means of their different activities on isolated

Table 1 The content of the pure PG compounds in a pool of human semen and in HSF PG extracted from the same pool The values have been estimated according to the method described by Bygdeman and Samuelsson (1964) and are expressed in μg per ml

PG compounds	Concentration in a pool of human semen n=3	Concentration in HSF PG extracted from the same pool n=3
PGE_1	15.5 (11.3—17.2)	12.3 (12.0—12.6)
PGE_2	12.8 (11.9—14.2)	6.6 (4.5—7.6)
PGE_3	2.5 (1.8—3.4)	1.2 (0.9—1.6)
$PGF_{1\alpha}$	2.0 (1.9—2.2)	1.3 (1.1—1.4)
$PGF_{2\alpha}$	2.2 (2.1—2.4)	1.5 (1.4—1.6)

rabbit jejunum ($F_1/E_2/E_3/F_{1\alpha}/F_{2\alpha}$=1/0.2/2/0.5/0.13), calculated from the values given in previous reports (Bergstrom and Euler 1963, Horton and Main 1963, Änggård and Bergstrom 1963, Bygdeman and Eliasson 1963 b, Bergstrom *et al* 1959 b, Samuelsson 1963 b) one unit of HSF PG corresponds to 7.5 μg of PGE_1 on this preparation

The sensitivity of the myometrium was measured in all experiments and expressed as ID_{50} units or μg per ml, i.e. the lowest amount tested that caused a decrease in the amplitude of the contractions by 50 per cent or more during at least 10 min. This was accomplished by adding increasing amounts of the substances starting with amounts that were below the probable threshold. Moreover, in those cases where the highest amount of prostaglandin tested did not cause an inhibition, this dose has been taken as ID_{50} in the statistical evaluation. This procedure is acceptable when the rank sum test by Dixon and Massey (1957) is used as in this investigation

Estimation of sodium, potassium and calcium

The estimation of sodium, potassium and calcium was performed using a flamephotometer (Eppendorf). The content of chloride was examined by chemical methods. The flamephotometer used was equipped with a spray chamber atomizer, a monochromator and a photomultiplier which made it suitable not only for sodium and potassium estimation but also for measuring of calcium (MacIntyre 1961). For the estimation of sodium and potassium a propane air flame and for calcium an acetylene air flame was used. The wave length was 589 $m\mu$, 766 $m\mu$ and 769 $m\mu$ and 620 $m\mu$ resp. Each estimation on the flame was made in duplicate

Distilled water always was used for dissolving or dilution Stock and standardsolutions were stored in polythene bottles All glassware was washed in hydrochloric acid and finally rinsed in distilled water

a) *Tyrode solution*

The Tyrode solution was estimated directly on the flame after diluting 200 times for sodium and 20 times for potassium and calcium The deflection on the flame graudated in mEqv per l was adjusted by a standard solution It was prepared from a stock solution containing 143 5 mEqv/l Na^+ 4 84 mEqv/l K^+ and 5 mEqv/l Ca^{++} by diluting 200 times for sodium and 20 times for potassium and calcium At the calcium estimation the zero point was adjusted with a solution containing the same concentration of sodium and potassium as the standard solution

b) *Myometrium*

The recorded pieces of myometrium were carefully dried immediately after the end of the recording and the wet weight was estimated The dry weight was measured after drying at 105°C for 72 hrs This time is sufficient for completely drying up human myometrium according to Cretius (1960) Even shorter time has been employed (Hawkins and Nixon 1958, Barnes and Kumar 1961) The weight loss corresponded to the total amount of water in the myometrium Then the myometrium was ashed for 16 hrs at 500°C For the quantity of myometrium used (200—300 mg) this time is sufficient to ash the myometrium piece completely without affecting its ion content (Grove Jones and Mathews 1961)

For the estimation of sodium potassium and calcium the myometrium was extracted with 2 ml 0 1 N warm hydrochloric acid According to Hawkins and Nixon (1958) this method should give slightly lower values for sodium and potassium than those obtained when using diluted nitric acid for the extraction With our technique however the recovery of added sodium and potassium was almost complete As calcium was estimated preferably on the same piece of myometrium as the other ions the use of hydrochloric acid seemed to be the most convenient method For the extraction of calcium diluted nitric acid is not sufficient (Hawkins and Nixon 1958)

The ion extract was diluted to 25 ml with distilled water Normally the same concentration range as that of the diluted stock solution used for the Tyrode solution estimation was reached In these cases the estimation

on the flame was made directly on the extract. If the content of either sodium, potassium or calcium was higher than 160, 5 and 6 mEqv/l respectively the extract was further diluted three times before the estimation.

The content of sodium in relation to potassium in the extract from the myometrium was about 10 times lower than that obtained in Tyrode solution. This was of importance for the calcium measurement. Therefore at the estimation of calcium in the myometrium a special stock solution had to be made containing 14.35 mEqv/l Na, 3.84 mEqv/l K^+ and 5.0 mEqv/l Ca. A corresponding standard solution was obtained by diluting the stock solution 20 times before use. The zero solution had the same concentration of sodium and potassium as the standard solution.

During the whole extraction process from immediately after recording until extraction with warm hydrochloric acid the myometrial pieces were kept in crucibels of known weight in order to avoid any loss of substance during weighing, drying or ashing procedures.

Estimation of chloride

The content of chloride in some of the recorded myometrial strips was estimated. All the determinations were made in duplicate. The hydrochloric extract was not suitable for chloride estimation. Therefore some of the strips from the same uterus were used for the ion and the rest for chloride estimation. The chloride was extracted from the myometrium by soaking in diluted nitric acid (3 ml 1 N HNO_3) for 7 days (Lowry and Hastings 1942). This method gives almost complete extraction as shown by Hawkin and Nixon (1958) for human myometrium. The content of chloride in the extract was estimated using the method described by van Slyke (1923). The chloride was precipitated as AgCl with $AgNO_3$ and excess silver was titrated with 0.02 N ammoniumthiocyanate using a microburette. $AgNO_3$ and concentrated HNO_3 were added separately according to the modification introduced by Wilson and Ball (1928).

Evaluation of the methods

a. *The estimation of ions with the flamephotometer*

A larger piece of myometrium was homogenized after drying and divided into several parts. The content of ions in these parts was measured separately. The variation found was normally not more than a few per cent (Table 2). To different parts of myometrium, made equal in the same way as described above, different amounts of a solution containing sodium,

Table 2 The table shows the reliability of the methods for estimation of sodium potassium calcium and chloride The values are given in mEqv per 100 g dry weight

	Uterus 1			Uterus 2		
	Na^+	K^+	Cl	Na^+	Ca^{++}	K^+
Mean	51.4	13.4	43.1	44.4	17.1	3.2
Range	50.6—52.1	13.1—13.6	40.9—46.8	42.4—48.0	16.9—17.5	3.1—3.3
Number of samples	n=3	n=3	n=4	n=4	n=4	n=4

potassium and calcium were added. The myometrium together with the added solution was then dried again, ashed and extracted with diluted hydrochloric acid. The amount of ions in the myometrium, in the added ion solution and in the myometrium together with the added ions was measured on the flame. The results showed that the method used gave complete or almost complete recovery for sodium and potassium and slightly lower values for calcium (Table 3).

Table 3 The table shows the recovery of added amounts of the ions sodium potassium and calcium The values are given in mEqv per 100 g dry weight

	Ions	Content of ions in the myo-	Added amount of ions	Found content of ions	Recovery in per cent Mean value
I	Na^+	42.2 (41.3—43.1) n=3	27.3	69.2 (68.9—69.4) n=4	99.5
I	K^+	21.0 (20.3—21.6) n=2	10.5	30.8 (29.3—31.5) n=4	97.6
I	Ca^{++}	2.9 (2.8—2.9) n=2	3.0	4.8 (4.6—5.9) n=4	81.6
II	Na^+	42.2 (41.3—43.1) n=3	53.4	96.6 (95.4—97.6) n=5	101.1
II	K^+	21.0 (20.3—21.6) n=2	19.2	40.4 (39.1—41.6) n=5	100.6
II	Ca^{++}	2.9 (2.8—2.9) n=2	6.0	8.0 (7.9—8.1) n=5	90.6

With the equipment used no important errors due to overlapping of emissions should occur in the sodium and potassium estimation. This was also found when solutions containing different amounts of sodium and potassium were tested. An increase of the concentration up to 4 times for either potassium, sodium or calcium did not affect the deflection on the flame more than 1—2 per cent for sodium and potassium. Somewhat different results were obtained when calcium was measured. If sodium or

on the flame was made directly on the extract If the content of either sodium potassium or calcium was higher than 160 5 and 6 mEqv/l respectively the extract was further diluted three times before the estimation

The content of sodium in relation to potassium in the extract from the myometrium was about 10 times lower than that obtained in Tyrode solution This was of importance for the calcium measurement Therefore at the estimation of calcium in the myometrium a special stock solution had to be made containing 14 35 mEqv/l Na^+, 3 84 mEqv/l K^+ and 5 0 mEqv/l Ca A corresponding standard solution was obtained by diluting the stock solution 20 times before use The zero solution had the same concentration of sodium and potassium as the standard solution

During the whole extraction process, from immediately after recording until extraction with warm hydrochloric acid the myometrial pieces were kept in crucibels of known weight in order to avoid any loss of substance during weighing drying or ashing procedures

Estimation of chloride

The content of chloride in some of the recorded myometrial strips was estimated All the determinations were made in duplicate The hydrochloric extract was not suitable for chloride estimation Therefore some of the strips from the same uterus were used for the ion and the rest for chloride estimation The chloride was extracted from the myometrium by soaking in diluted nitric acid (3 ml 1 N HNO_3) for 7 days (Lowry and Hastings 1942) This method gives almost complete extraction as shown by Hawkins and Nixon (1958) for human myometrium The content of chloride in the extract was estimated using the method described by van Slyke (1923) The chloride was precipated as AgCl with $AgNO_3$ and excess silver was titrated with 0 02N ammoniumthiocyanate using a micro burette $AgNO_3$ and concentrated HNO_3 were added separately according to the modification introduced by Wilson and Ball (1928)

Evaluation of the methods

a) *The estimation of ions with the flamephotometer*

A larger piece of myometrium was homogenized after drying and divided into several parts The content of ions in these parts was measured separately The variation found was normally not more than a few per cent (Table 2) To different parts of myometrium, made equal in the same way as described above different amounts of a solution containing sodium,

None of these substances is ideal for measuring the extracellular space. Normally the values for chloride and sodium space are higher than those obtained with inulin (Kao 1961, Daniel and Daniel 1957, Bozman et al. 1959). On rabbit myometrium, however, Horvath (195[illegible]) found similar values for inulin, chloride and sodium space. One reason for the lower inulin space is that inulin does not evenly penetrate the extracellular space (Nichols, Nichols, Weil and Wallace 1953, Cotlove 1955). The magnitude of inulin space varies widely not only in samples of myometrium from different animals but also in different samples from the same uterine horn in rabbits (Kao 1961). If inulin is added in vitro continuously for a long time, the inulin and chloride space will become almost equal in rat muscle (Cotlove 1952). The chloride ion naturally does not have this disadvantage, as it is normally always in equilibrium with its surroundings. Also chloride will, however, give no exact values for the extracellular space. If the sodium distribution is calculated using the chloride space, sometimes negative values are obtained for the intracellular sodium concentration in human myometrium, indicating that some chloride ions are intracellular (Daniel 1958, Daniel and Boyes 1957). This is also valid for striated muscle (e.g. Conway 1957).

In this investigation chloride space was chosen for calculation of ion distribution inside and outside the cell. This choice made it possible to compare some of the results obtained with some of the more recent investigations on ion content and ion distribution in human myometrium (Hawkins and Nixon 1958, Cort and Cort 1957, Barnes and Kumar 1961).

It was assumed that strips taken from one uterus at the same place had the same extracellular volume as measured with chloride space. For simplicity the term "extracellular space" has been used as though it was equal to the chloride space.

Using chloride space for estimation of extracellular space is incorrect when the intracellular concentration of chloride varies. This error affects the ion values to a different degree. Since the chief part of potassium is found intracellularly, even wide errors in extracellular space estimation affect the values very little. Similar conditions apply to calcium and magnesium, which are also largely found intracellularly. On the other hand, the major part of sodium in tissue is found extracellularly and therefore even small errors in extracellular space estimation can lead to large errors in the intracellular sodium values.

The extracellular space was calculated according to Hastings and Eichelberg (1937) and Kühn (1934). In the calculation of the ion con-

centration in extracellular fluid a correction was made for the Donnan factor (0.96)

Abbreviations used

Cl_T = mEqv chloride per liter Tyrode solution
Cl_M = mEqv chloride per 100 g dry muscle
Na_T = mEqv sodium per liter Tyrode solution
Na_e = mEqv extracellular sodium per 100 g dry muscle
H_2O_e = g or ml extracellular water per 100 g dry muscle
H_2O_t = g or ml water per 100 g dry muscle

The content of water in the chloride space was calculated as follows

$$H_2O_e = \frac{Cl_M \quad 1000}{Cl_T \quad 1.04} \text{ g per 100 g dry muscle}$$

The sodium concentration in the extracellular space is

$$Na_e = \frac{Na_T \quad H_2O \quad 0.96}{1000} \text{ mEqv per 100 g dry weight}$$

The concentration of the other ions was calculated in a similar way The intracellular water content and the concentration of the ions were obtained by subtracting the extracellular values from the total

THE EFFECT OF HSF PG, PGE$_1$, PGE$_2$, PGE$_3$, PGF$_{1\alpha}$ AND PGF$_{2\alpha}$ ON HUMAN MYOMETRIUM

Human seminal fluid normally inhibited the spontaneous motility of human non pregnant myometrium *in vitro* Stimulation was however found in a few cases (Kurzrok and Lieb 1930, Cockrill *et al* 1935) A crude extract of human semen, HSF PG, gave a similar inhibitory effect when studied on 8 myometrial strips (Eliasson 1959) The results from the same investigation also indicated that the myometrium was most sensitive to HSF-PG at ovulation time

With regard to the important proposed role of prostaglandin for normal fertility the study of the effect of HSF PG was extended in this work and the effect of the pure PG compounds, PGE$_1$ PGE$_2$ PGE$_3$ PGF$_{1\alpha}$ and PGF$_{2\alpha}$ present in human semen (Samuelsson 1963 b) was examined Special attention was paid to variations in sensitivity of the myometrium during the menstrual cycle.

RESULTS

The material was divided into groups according to the hormonal influence Group 1 corresponds to early middle proliferative phase (approx 1—10 day), group 2 to late proliferative phase (approx 10—14 day) group 3 to early secretory phase (approx 14—20 day), group 4 to middle late secretory phase (approx 20—28 day), group 5 to menopause and group 6 to metropathia hemorrhagica cystica (MHC) The division was made by

Table 5 Number of strips and uteri in the different groups when HSF PG was tested

Hormonal phase	Groups						
	1 Early middle *proliferative* phase	2 Late proli *ferative* phase	3 Early *secretory* phase	4 Middlelate *secretory* phase	5 Meno- *pause*	6 MHC	7 Pregnancy
No of uteri	17	14	12	8	4	5	10
No of strips	50	30	32	19	14	12	40

histological examination of the endometrium performed by Dr A Lindgren, and correlated with the history of menstrual bleedings Strips from pregnant uteri both in early and late pregnancy were placed in group 7

The effect of HSF PG on human myometrium

In this part 197 strips from 70 uteri were used The distribution between the different groups is illustrated in Table 5

a) *Non pregnant myometrium*

An inhibition of the spontaneous motility following the addition of HSF PG was obtained in 90 per cent of the non pregnant strips The dose of prostaglandin needed for an ID$_{50}$ effect varied, however, in the different groups It was found that the myometrium is most sensitive around ovulation time, e g group 2 and 3 Usually 0 003 unit per ml bath fluid is sufficient to produce an effect (Fig 4 and 5) Fig 4 also illustrates the dose response relationship During the other phases of the menstrual cycle the sensitivity to HSF PG is much less, and some preparations did not respond even to the highest doses used (0 5 unit/ml)

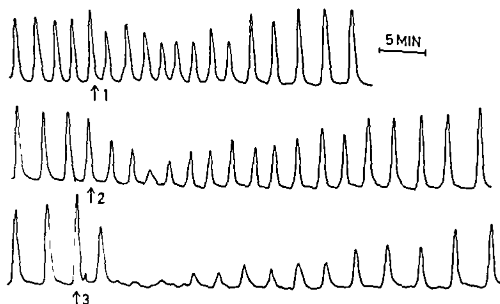


Fig 4 The dose response of the isolated human myometrium to HSF PG The myometrium is in late proliferative phase 1 = 0 003 U/ml 2 = 0 01 U/ml 3 = 0 03 U/ml 0 01 U ~ 0 03 µg PGE_1 and 0 004 µg $PGF_{1\alpha}$

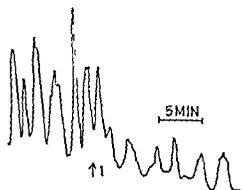


Fig 5 The effect of 0 003 U HSF PG ml on an isolated strip from human myometrium in early secretory phase 0 003 U ~ 0 01 μg PGE_1 and 0 001 μg $PGF_{1\alpha}$

(Fig 6) The sensitivity of the myometrium is 3—5 times higher at ovulation time than early and late in the menstrual cycle

The variation in sensitivity of the myometrium to HSF PG under various conditions is illustrated in Tables 6, 7 and 8 The figures in Table 6 are calculated from the median values for each uteri which are based on records from 2 to 4 strips From the statistical evaluation (Table 7) it is apparent that the difference in sensitivity between uteri from mid cycle and those from early and late in the menstrual cycle is highly significant

The sensitivity of the myometrium taken from patients who are in menopause has also been tested, although the 14 strips from 4 uteri which are included in this group do not allow a statistical analysis of the



Fig 6 The lack of effect of HSF PG on the motility of isolated human myometrium in late secretory phase 1=0 033 U/ml 0 033 U ~ 0 1 μg PGE_1 and 0 012 μg $PGF_{1\alpha}$

Table 6 Variations in sensitivity of pregnant and non pregnant human myometrium to HSF PG in vitro

Group	No of uteri	Hormonal status	HSF PG ID_{50} (units/ml)	
			Median values	Range
1	17	Early middle proliferative phase	0 015	0 0025—0 05
2	14	Late proliferative phase	0 0045	0 001 —0 01
3	12	Early secretory phase	0 007	0 0025—0 03
4	8	Middle late secretory phase	0 022	0 01 —0 15
6	5	MHC	0 01	0 003 —0 035
7	10	Pregnancy	0 04	0 003 —0 1

Table 7 Statistical significance in the difference of sensitivity between the various groups of uterine strips to HSF PG The group numbers correspond to those in Table 6

Comparison between groups	Degree of significance (P value)
1 and 2	0 01—0 001
2 and 3	No difference
2 and 4	<0 001
1 and 4	No difference

Table 8 Variation in sensitivity of human myometrium to HSF PG in relation to time after the onset of the menopause

Years after the last normal menstruation	Numbers of experiments		Mean value of HSF PG ID_{50} (units/ml)
	Uteri	Strips	
1	1	4	0 0095
2	1	4	0 005
4	1	2	0 03
5	1	4	0 075

results It is, however, of interest that a decrease in sensitivity with time after the onset of the menopause was observed (Table 8)

Strips taken from patients with disturbances in the hormone production of type MHC (group 6) usually have low sensitivity to HSF-PG All

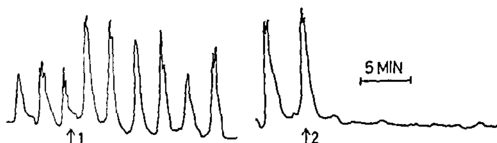


Fig 7 The effect of HSF PG on the motility of isolated human myometrium in late proliferative phase 1 = 0 0007 U/ml 2 = 0 003 U/ml 0 003 U ~ 0 01 μg PGE_1 and 0 001 μg $PGF_{1\alpha}$

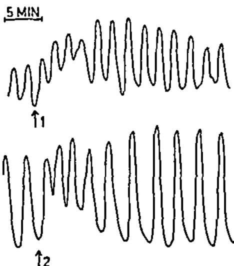


Fig 8 The effect of HSF PG on the motility of isolated human myometrium in early proliferative phase Note the stimulation even when a larger dose is added 1 = 003 U/ml 2 = 0 015 U/ml 0 015 U ~ 0 05 μg PGE_1 and 0 006 μg $PGF_{1\alpha}$

strips responded with inhibition and the median value for ID_{50} wa *0 01* units per ml (range *0 003—0 035*)

Some uteri preparations were stimulated by HSF PG It further appeared that these uteri could be divided into two sub-groups since some of them responded with the ordinary inhibition to larger doses of HSF PG while others were stimulated by all doses used This is illustrated in Fig 7 and 8 In Fig 7 the stimulatory effect of a very small dose of HSF PG on an uterine strip from late proliferative phase is shown The addition of a *slightly larger dose of HSF PG caused a marked inhibition The second* type of reactivity pattern is illustrated in Fig 8 Both a moderate and a high dose of HSF PG caused an increase in tonus and amplitude of the contractions This reactivity pattern was observed in 16 strips from 6 uteri, equally distributed between groups 1, 3 and 4

b) *Pregnant myometrium*

The effect of HSF PG on pregnant myometrium has been studied on 40 strips from 10 uteri Nine uteri were from the 14th to 20th week of gestation and one a full term pregnancy The normal effect of HSF PG on isolated pregnant myometrium is that of stimulation although inhibition was registrated in some cases A dose of about *0 025 to 0 05 units of* HSF PG per ml bath fluid stimulated the spontaneous motility in 7 out of 10 investigated uteri, including the full term pregnancy (Fig 9 and 10) The stimulation was however, not linear to increasing doses of HSF PG The stimulation was similar to that obtained by oxytocin In two

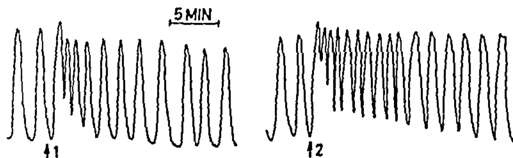


Fig 9 The effect of HSF PG (1=0 07 U/ml) on an isolated strip from human myometrium during late pregnancy in comparison with oxytocin (2=1 mU/ml) 0 07 U ~ 0 2 μg PGE$_1$ and 0 028 μg PGF$_{1\alpha}$

of the three remaining experiments the strips reacted with inhibition to HSF PG The ID_{50} dose was 0 003 and 0 025 units per ml bath fluid

Both the dose of HSF PG, which gives an inhibition and the dose necessary for a stimulation is high compared with the doses used for the non pregnant myometrium, at least at ovulation time

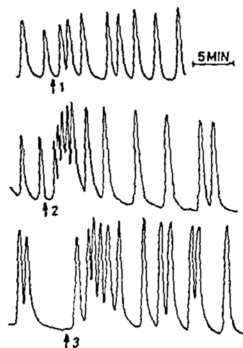


Fig 10 The effect of HSF PG on human pregnant myometrium (15th week of pregnancy) in comparison with oxytocin 1=0 005 U/ml and 2=0 05 U/ml HSF PG 3=1 mU/ml oxytocin 0 05 U ~ 0 15 μg PGE$_1$ and 0 02 μg PGF$_{1\alpha}$

The effect of PGE_1, PGE_2, PGE_3, $PGF_{1\alpha}$ and $PGF_{2\alpha}$ on human myometrium

The effect and sensitivity of the myometrium to the different prostaglandin compounds has been tested on 233 strips from 45 both pregnant and non pregnant uteri. The material has been divided into groups after the same principle as for HSF PG and the distribution is illustrated in Table 9.

Table 9. Number of strips and uteri in the various groups when the different prostaglandins were tested

Hormonal phase	Groups 1 Early middle proliferative phase	2 Late proliferative phase	3 Early secretory phase	4 Middle late secretory phase	7 Pregnancy
PGE_1 Strips/uteri	62/13	68/13	11/4	32/8	24/7
PGE_2 & PGE_3 Strips/uteri		2/1	2/1	4/2	
$PGF_{1\alpha}$ & $PGF_{2\alpha}$ Strips/uteri	4/4	5/5	4/4	3/3	12/4

a) *Non pregnant myometrium*

The normal effect of PGE_1 *in vitro* is an inhibition of the spontaneous motility of the myometrium. This inhibition is characterized by a decrease in the amplitude and frequency of contractions.

In late proliferative phase normally (in 11 out of 13 uteri) 0.01—0.05 ng per ml bath fluid gave an ID_{50} inhibition of the motility (Fig. 11). Two uteri in this group were abnormally insensitive. 0.1 µg had to be given in order to obtain an ID_{50} inhibition. The sensitivity during the early secretory phase was similar to that found in late proliferative phase although the number of uteri in this group is too small to allow a statistical evaluation.

In early middle proliferative and middle late secretory phase the sensitivity of the myometrium to PGE_1 was lower, about half of that found in the other groups if median values are compared. The effect of PGE_1 on a strip from late secretory phase is illustrated in Fig. 12. Median values and dose range for PGE_1 are given in Table 10. It is worth noticing that the lowest dose 0.02 µg was obtained when testing a uterus from the 21st day of the menstrual cycle. The difference in sensitivity to PGE_1

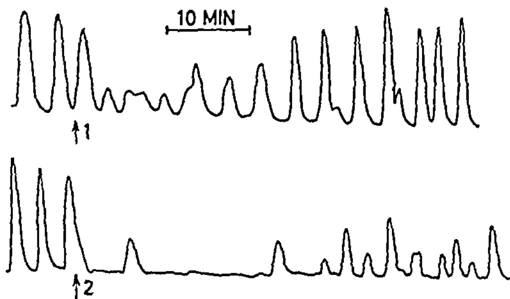


Fig 11 The effect of PGE_1 on an isolated strip from human myometrium in late proliferative phase 1 = 0.01 μg/ml 2 = 0.03 μg/ml

Fig 12 The effect of PGE_1 on an isolated strip from human myometrium in late secretory phase 1 = 0.03 μg/ml 2 = 0.1 μg/ml

Table 10 Variations in sensitivity of pregnant and non pregnant human myometrium to PGF_1 in vitro

Groups	No of uteri	Hormonal status	PGF_1 ID_{50} (μg/ml) Median values	Range
1	13	Early middle proliferative phase	0.05	0.03—0.1
2	13	Late proliferative phase	0.025	0.01—0.1
3	4	Early secretory phase	0.025	0.01—0.05
4	8	Middle late secretory phase	0.05	0.02—0.5
7	7	Pregnancy	0.05	0.03—0.3

Table 11 Statistical significance in the difference of sensitivity between various groups of uterine strips to PGE_1 The group numbers correspond to those in Table 9

Comparison between groups	Degree of significance (P value)
1 and 2	0.01—0.001
2 and 4	0.05—0.01
1 and 4	No difference

between uteri from ovulation time and those from other phases of the menstrual cycle is statistically significant (Table 11)

In some experiments (8 strips from 4 uteri) the effect of PGE_2 and

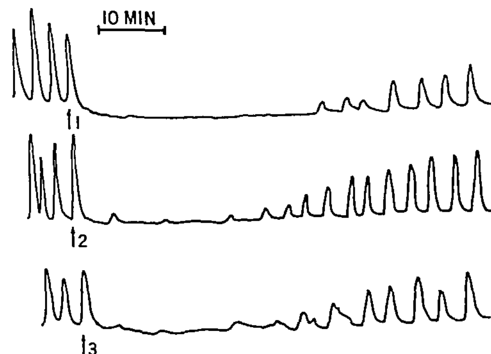


Fig 13 The effect of PGE_1 PGE and PGE_3 on human isolated myometrium The strips are from an uterus in middle secretory phase 1=0.03 μg/ml PGE_1 2=0.03 μg/ml PGE 3=0.03 μg/ml PGE_3

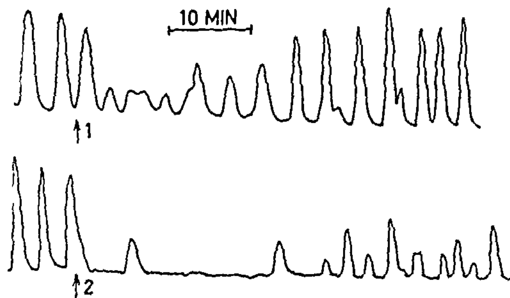


Fig 11 *The effect of PGF_1 on an isolated strip from human myometrium in* late proliferative phase 1=0.01 µg/ml 2=0.03 µg/ml

10 MIN

↑1

↑2

Fig 12 *The effect of PGF_1 on an isolated strip from human myometrium in late secretory phase* 1=0.03 µg/ml 2=0.1 µg/ml

Table 10 Variations in sensitivity of pregnant and non pregnant human myometrium to PGE_1 in vitro

Groups	No of uteri	Hormonal status	PGE_1 ID_{50} (μg ml)	
			Median values	Range
1	13	Early middle proliferative phase	0 05	0 03—0 1
2	13	Late proliferative phase	0 025	0 01—0 1
3	4	Early secretory phase	0 025	0 01—0 05
4	8	Middle late secretory phase	0 05	0 02—0.5
7	7	Pregnancy	0 05	0 03—0.3

Table 11 Statistical significance in the difference of sensitivity between various groups of uterine strips to PGE_1 The group numbers correspond to those in Table 9

Comparison between groups	Degree of significance (P value)
1 and 2	0 01—0 001
2 and 4	0 05—0 01
1 and 4	No difference

between uteri from ovulation time and those from other phases of the menstrual cycle is statistically significant (Table 11)

In some experiments (8 strips from 4 uteri) the effect of PGE_2 and

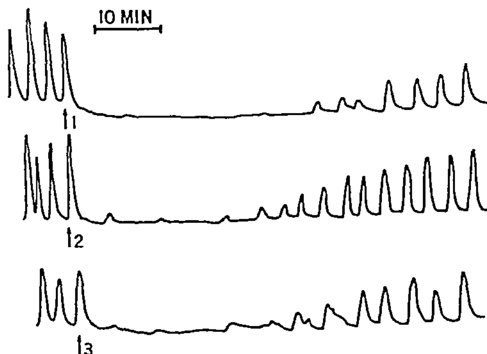


Fig 13 The effect of PGE_1 PGE_2 and PGE_3 on human isolated myometrium The strips are from an uterus in middle secretory phase $1=0\,03$ μg ml PGE_1 $2=0.03$ μg ml PGE $3=0\,03$ μg ml PGE_3

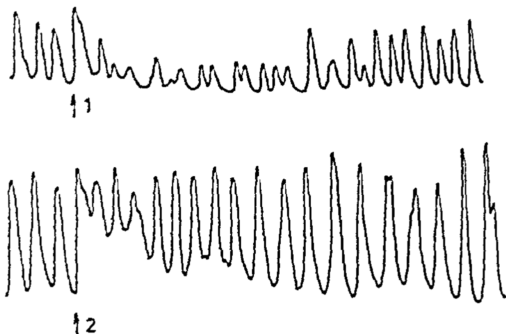


Fig. 14 The effect of 0.05 μg/ml PGE₁ (1) and 0.5 μg/ml $PGF_{1\alpha}$ (2) on two strips from an uterus in late proliferative phase

PGE₂ was tested. The effect of these substances is similar to that found for PGE_1 (Fig. 13) although the sensitivity of the myometrium is slightly lower. If mean values are compared 1 μg of PGE_1 corresponds to 1.3 μg of E_2 and to 1.5 μg of E_3. Normally 0.02—0.3 μg of E_2 and 0.02—0.3 μg of E_3 give an ID_{50} inhibition.

The effect of the two PGF-compounds tested ($PGF_{1\alpha}$ and $PGF_{2\alpha}$) differs from that of the PGE-group. In the same dose range, 0.01—0.1 μg where PGE_1 gives an ID_{50} inhibition, the F-compounds are without effect. An increased dose, 0.3—0.5 μg/ml, usually gives for $F_{1\alpha}$ (in 6 uteri out of 12) a stimulation of the motility characterized primarily by an increase in tonus (Fig. 14). On the remaining uteri this dose was without effect in five and gave an inhibition in one. This uterus was from late proliferative phase.

b. *Pregnant myometrium*

The effect of PGE_1 was tested on 24 strips from 7 pregnant uteri and that of $PGF_{1\alpha}$ on another 12 strips from 4 of the 7 uteri (Table 9). Six uteri were from early pregnancy (12th to 20th week). The last one was a full term pregnancy. In early pregnancy the effect of PGE_1 was somewhat complicated. In about half of the uteri 0.01 to 0.03 μg PGE_1 per ml

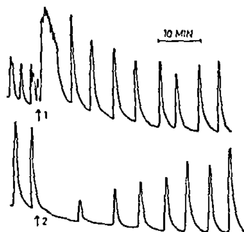


Fig 15 The effect of PGE_1 on a strip from human myometrium during the fourth month of pregnancy 1=0 01 µg/ml 2=0 03 µg/ml

bath fluid increased the tonus Later during the recording 0 03—0 1 µg, however, gave the usual inhibition (Fig 15) found for the nonpregnant myometrium In the remaining experiments 0 01 µg was without effect but 0 03 µg decreased the motility In the full term pregnancy case 0 01 to 0 3 µg was without any effect at all

$PGF_{1\alpha}$ always stimulates the motility of pregnant isolated myometrium in the same way as found for non pregnant myometrium although the sensitivity of the strips is increased Even a dose of 0 01 µg per ml bath fluid is enough and the stimulatory effect remains even if the dose is increased to 0 5 µg (Fig 16)

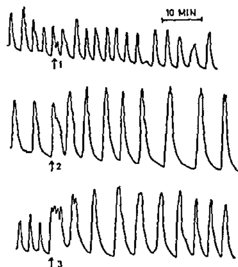


Fig 16 The effect of $PGF_{1\alpha}$ on human isolated myometrium from an uterus in the fourth month of pregnancy 1=0 01 µg/ml 2=0 03 µg/ml 3=0 3 µg/ml

DISCUSSION

Normally all PGE compounds and HSF PG inhibit the spontaneous motility of human myometrium The effect of all the actual PG compounds was described in a preliminary report (Eliasson and Bygdeman 1963 b) The same year reports confirming the results for PGE_1 (Sandberg *et al* 1963 b) and for PGE_2 (Pickles and Hall 1963) were published

Contrary to the PGE compounds both $PGF_{1\alpha}$ and $PGF_{2\alpha}$ have a tendency to stimulate the spontaneous motility of human non pregnant myometrium The effect of $PGF_{2\alpha}$ has also been confirmed (Pickles and Hall 1963)

An inhibitory effect on human myometrium of an autopharmacological active substance is uncommon The only substance with such an effect in vitro seems to be bradykinin (Landesman, Campell and Wilson 1963)

The sensitivity of human myometrium to PGE_1 is of the same order as that of some other smooth muscle preparations e g guinea pig ileum, rat uterus and rabbit jejunum (Bergstrom and Euler 1963, Horton and Main 1963) The ID_{50} dose for PGE_1 on human myometrium, 0 03 μg per ml bath fluid, is of the same magnitude as for other autopharmacological active substances e g acetylcholine 0 5—1 0 μg/ml, adrenaline 0 5—1 0 μg/ml (Sandberg *et al* 1958), vasopressin 0 001—0 003 μg/ml (Bygdeman and Eliasson 1963 d) and bradykinin 0 2—1 0 μg/ml (Landesman *et al* 1963)

The sensitivity of the myometrium to PGE_1 and to HSF PG varies in a characteristic way during the different phases of the menstrual cycle The activity is most pronounced around ovulation time and less during the other phases of the menstrual cycle It can be expected that the same condition is also valid for PGE_2 and PGE_3 The fact that HSF PG, which contains all three E compounds shows the same variation somewhat more pronounced advocates the assumption

HSF PG contains PGE_1, PGE_2 PGE_3 $PGF_{1\alpha}$ and $PGF_{2\alpha}$ It can therefore be expected that the effect of HSF PG should correspond to their total effect On human myometrium all PGE compounds have the same inhibitory effect and all PGF compounds the same stimulating effect It is therefore justified, and makes the comparison between the effect of the individual compounds and HSF PG easier, to take PGE_1 and $PGF_{1\alpha}$ as representatives for each group From the estimated values of HSF PG (expressed in units) on isolated rabbit jejunum and of the different prostaglandin compounds (expressed in μg) by using the method

of Bygdeman and Samuelsson (1964) it is possible to compare the doses given of HSF PG with those of PGE_1 and $PGF_{1\alpha}$. On human myometrium 1 unit of HSF PG corresponds to 3 μg of PGE_1 and to 0.4 μg of $PGF_{1\alpha}$. On rabbit jejunum the corresponding value for PGE_1 was 7.5 μg. The lower value calculated for human myometrium chiefly depends on the difference in the relative activity of PGE_2 in comparison with PGE_1. On rabbit jejunum 1 μg of PGE_2 corresponds to about 5 μg of PGE_1. On human myometrium the relationship is almost 1 to 1. For the effect of HSF PG on non pregnant myometrium the content of $PGF_{1\alpha}$ and $PGF_{2\alpha}$ does not play any essential role as even a relative large dose of HSF PG, i.e. 0.03 units contains only about 0.012 μg of $PGF_{1\alpha}$ and $PGF_{2\alpha}$ together. The threshold dose for the substances under this condition is normally about ten times higher.

It is therefore clear that the effect of HSF PG on non pregnant myometrium depends on its content of PGE compound. In the material, where HSF PG was tested on 197 strips from 70 uteri and PGE_1 on 207 strips from 45 uteri a good correspondence between the effect of a certain amount of HSF PG and that of suitable amounts of PGE_1 as a representative for the PGE group was found. If for instance the median ID_{50} values for PGE_1 and HSF PG during the different phases of the menstrual cycle are compared one unit of HSF PG corresponds to 2.3—5.0 μg of PGE_1 (mean 3.5 μg). This relationship 1 unit $\sim$ 3.5 μg well resembles the calculated one, 1 unit $\sim$ 3 μg.

In the individual case however some differences exist. In a few experiments all doses of HSF PG stimulated the spontaneous motility. The same result was not found for PGE_1 on non pregnant uteri. It is possible that an extended study on the effect of PGE_1 also will include uteri which are stimulated by PGE_1.

Kurzrok and Lieb (1930), and Cockrill *et al.* (1935) demonstrated that human seminal fluid usually caused an inhibition of the spontaneous motility of the isolated human myometrium. In a few cases instead the semen had a stimulatory effect. This effect well resembles that of HSF PG. It is interesting that they have the same rate of stimulation (10%) as found here for HSF PG. The different effect of human semen may however be due to variations in the content of the different PG compounds in the samples.

On pregnant myometrium the content of the PGF compounds plays a more important role for the effect of HSF PG than on non pregnant myometrium. Here the stimulatory threshold dose for $PGF_{1\alpha}$ and $PGF_{2\alpha}$ is well reached with the amount of HSF PG used (0.025—0.05 units of

HSF PG corresponds to 0 01—0 02 μg of $PGF_{1\alpha}$ and $PGF_{2\alpha}$ together) Also the dual effect of PGE_1 will be of importance The effect of HSF PG therefore depends on the actual effect of the two prostaglandin groups In most experiments, however, HSF PG stimulated the spontaneous motility The stimulation was sometimes not pronounced by an increased dose The explanation might be that an initial low dose of HSF PG stimulates the motility due to the stimulatory effect of the PGF and eventually also of the PGE compounds A later higher dose of HSF PG has less effect as now only the PGF compounds stimulate while the PGE s instead inhibit the motility

The effect of the crystalline compounds on human uterine motility *in vivo* has not yet been investigated The effect of HSF PG *in vivo* however, corresponds to that found *in vitro* as demonstrated by Eliasson and Posse (1960) They instilled HSF PG intravaginally at the calculated ovulation time in fertile women and studied the uterine motility with the technique described by Karlson (1944) Karlson used three separate pressure transducers which simultaneously recorded the pressure changes in the corpus isthmus and cervix uteri HSF PG caused at this time of the cycle, after a latency period during which HSF PG was postulated to be absorbed from the vagina an inhibition of the spontaneous motility In other phases of the menstrual cycle the uterus was much less sensitive to HSF PG and the effect irregular If the sensitivity of the myometrium at ovulation time was increased by simultaneous intravenous infusion of post pituitary hormones intravaginal application of HSF PG evoked a more consistent and marked inhibitory response

CHAPTER IV

THE EFFECT OF VARIATIONS IN THE EXTRACELLULAR ION CONCENTRATION ON THE SPONTANEOUS MOTILITY AND CONTENT OF IONS OF HUMAN MYOMETRIUM

The sensitivity of human myometrium to the PGF compounds and HSF PG varies during the different phases of the menstrual cycle The factors which regulate the reactive ability of human isolated myometrium are not known Some authors (e g Csapo 1954 1956 Marshall and Csapo 1961, Jung 1959) working with myometrial preparations from rabbit and rat have however stressed the importance of ion gradients especially the intra and extracellular relationship between cations for the reactivity of the myometrium Variations in the extracellular ion concentration could profoundly change the reactivity pattern of the myometrium A high extra cellular potassium concentration for instance decreased the membrane potential increased the frequency of the contractions and increased the sensitivity of the myometrium to different stimuli

Csapo (1959) has also demonstrated that an estrogen dominated rabbit uterus reacts in a quite different way than a progesterone dominated one to electrical stimulation It was of special interest that the reactivity pattern of a progesterone dominated uterus could be simulated by treating the estrogen dominated muscle *in vitro* with a Krebs solution containing excess K and low Na^{+} (Csapo 1959)

The effect of extracellular ion variations on the ion content, motility and reactivity pattern of human myometrium does not seem to have been investigated before Therefore the effect of low and high extracellular potassium and low extracellular calcium concentration on human myometrium was first examined with regard to motility and ion content in order to clarify if human myometrium reacts in a similar way as the animal myometrial preparations

SPECIAL METHODS

The extracellular concentrations of potassium and calcium have been varied The strips were first allowed to stabilize in normal Tyrode solution

and the ion concentration was slowly changed, for potassium from the normal value 5 6 ± 0 1 mEqv/l up to 16 3 ± 0 3 mEqv/l and down to 1 6 ± 0 1 mEqv/l and calcium from 3 0 ± 0 2 mEqv/l to 1 0 ± 0 1 mEqv/l In some experiments the potassium concentration was increased to 8 6 ± 0 1 mEqv/l and 11 5 ± 0 1 mEqv/l For all values the variation is given as S E M The amplitude and frequency were measured during several 10 minute periods, before and after the change in ion concentration At the end of the experiment the content of potassium, sodium, calcium and chloride was estimated in the recorded strips Further, pieces of non recorded myometrium were also examined for their ion content

Normally, four strips were used from the same uterus One was exposed to high potassium the second to low potassium, and the third to low calcium concentration The fourth strip was used as control e g no change in the ion concentration was performed On this strip the stability of the motility during the experimental time and the ion content of myometrium in normal Tyrode solution could be studied

RESULTS

A *The effect of extracellular ion variations on the spontaneous motility of isolated myometrium*

Normally the amplitude slightly increased and the frequency decreased in the control bath during the time of the experiment The difference in motility of the control strips and the strips in the experimental baths is presented as mean values ± S E M

a *Potassium*

The material in this group consisted of 34 strips from 17 uteri Another 23 strips from the same 17 uteri were used as controls An increase in the extracellular potassium concentration influenced the motility in a characteristic continuous manner The frequency increased as well as the tonus in most of the experiments (Fig 17 and 18) The amplitude decreased both in absolute figures and in relation to the control bath At the highest potassium concentration used, 16 3 mEqv/l, the increase in frequency was 3 4 ± 0 5 contractions per 10 min The amplitude of the contractions decreased by about 25% Absolute figures for the amplitude is difficult to give as the individual variation sometimes is rather pronounced

A decrease in the potassium concentration to 1 6 mEqv/l gives a less pronounced opposite effect on the spontaneous motility The frequency decreased by 0 6 ± 0 3 contractions, while the amplitude compared with

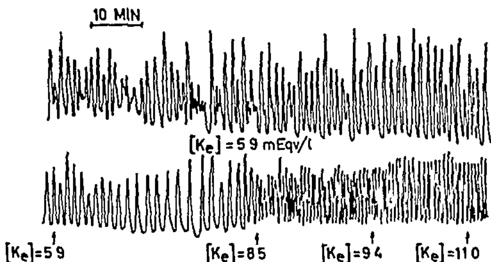


Fig 17 The effect of a contineous increase in the [K] concentration on the spontaneous motility of an isolated strip (lower curve) in comparison to a control strip from the same uterus (upper curve)

control strip remained almost unchanged (Fig 19) The results are summarized in Fig 20

b *Calcium*

An extracellular decrease in the calcium concentration from the normal 3 0 mEqv/l to 1 0 mEqv/l was examined on 22 strips from 16 uteri with the same number of controls The effect on spontaneous motility, due to diminished calcium concentration is a decrease in frequency by 0 7 ± 0 3 contractions for a 10 minutes period and a slightly lowered amplitude

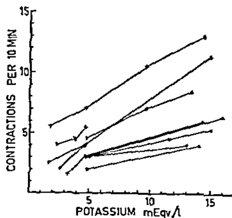


Fig 18 The effect of variations in the extracellular potassium concentration on the frequency of the spontaneous motility

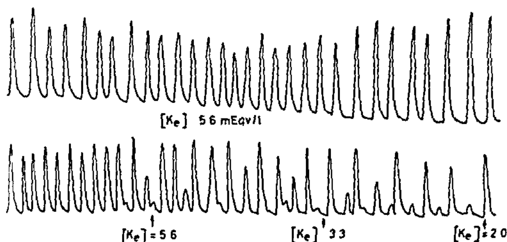


Fig 19 The effect of a continuous decrease in the [K] concentration on the spontaneous motility of an isolated strip (lower curve) in comparison to a control strip from the same uterus

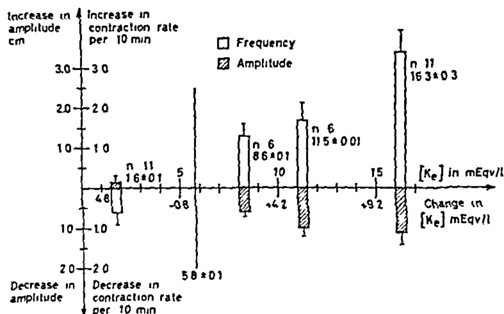


Fig 20 The effect of changes in the potassium concentration on the spontaneous motility of human myometrium *in vitro*

B *The effect of extracellular ion variation on the ion content of isolated myometrium*

The ion content of non recorded myometrium was examined on 131 strips from 25 uteri and that of the recorded myometrium on 112 strips from 26 uteri The distribution of the uteri between the different groups earlier defined is presented in Table 12 If not otherwise stated, all values for the myometrial content of water and ions are expressed in g and mEqv per 100 g dry weight respectively The values given for the ion content are mean values and the variation (if the material exceeded 5 uteri) is expressed as S E M

The total ion content in human pregnant and non pregnant myometrium under different conditions is summarized in Table 12 The non recorded myometrium shows only small differences in its content of water, sodium potassium calcium and chloride during the different phases of the menstrual cycle Pregnant myometrium contains however, more water sodium and chloride than the non pregnant myometrium

If the myometrium is exposed to Tyrode solution the ion content of the strips is different from that found in non recorded strips although the motility seems regular and normal The sodium concentration has increased by 23 6 mEqv and the potassium concentration decreased by 9 6 mEqv As the water content is relatively constant the same differences exist even if the values are calculated in relation to wet weight In order to obtain osmotic equilibrium one has to assume that the excess of sodium is bound somewhere in the cell The chloride content also increases but only so much that it corresponds to the higher chloride content in Tyrode solution compared to plasma For pregnant myometrium the sodium and chloride content also increases If the material for the recorded strips in Tyrode solution is divided into groups according to the phase in the menstrual cycle no differences are found

Changes in the extracellular calcium and potassium concentration affected the ion content of the strip An increase in the potassium concentration of the bath fluid from 5 6 mEqv/l to 15 2 mEqv/l raises the potassium content from 17 9 to 24 1 mEqv Although the potassium concentration in the bath fluid is raised three times the content of potassium in the non recorded strips is not reached A decrease to 1 8 mEqv/l lowers the content to 15 8 mEqv That the sodium values for the 8 6 and 11 5 mEqv uteri slightly differ from the remaining ones probably depends on the fact that two experimental series with different uteri were performed The first one contained the 8 6 and 11 5 mEqv potassium experiments and the second

Table 12 The content of water and ions sodium potassium calcium and chloride in the non recorded strips and the strips registrated in Tyrode with different potassium and calcium concentrations Mean values are given ± standard error of mean (if more than 5 uteri)

Different groups according to the hormonal status		H_2O g/100 g	Na mEqv/100 g	K mEqv/100 g	Ca mEqv/100 g	Cl mEqv/100 g	Number of examined strips and uteri
Non recorded strips	Group 1	397±7	42.5±1.5	26.5±1.5	3.8±1.1	32.2±2.1	30/6
	Group 2+3	396±6	42.7±1.0	28.0±0.8	3.7±0.3	32.7±2.5	59/10
	Group 4	396±11	41.0±1.5	27.8±1.0	3.8±0.3	29.3±1.2	26/6
	Group 1—4	396±9	42.1±1.4	27.5±1.0	3.8±0.5	31.5±2.1	115/22
	Group 7	437	53.8	24.2	4.1	18.6	8/3
Strips recorded in Tyrode	Group 1—4	399±14	65.0±2.2	17.9±0.7	3.7±0.2	14.3±1.9	22/11
	Group 7	425	71.7	20.5	4.1		10.5
Strips recorded in Tyrode with $[K_e]$ 1.8±0.1 mEqv/l		400±9	65.0±1.5	15.8±0.9	3.9±0.1	12.9±2.9	20/10
Strips recorded in Tyrode with $[K_e]$ 8.6±0.1 mEqv/l		379±5	58.1±1.5	21.9±0.2	4.2±0.5	38.8±2.2	12.6
Strips recorded in Tyrode with $[K_e]$ 11.5±0.1 mEqv/l		376±10	59.9±1.9	22.3±0.7	4.1±0.2	38.9±1.8	12/6
Strips recorded in Tyrode with $[K_e]$ 15.2±1.1 mEqv/l		408±9	64.7±0.6	24.1±0.9	3.9±0.7	15.1±2.1	20/10
Strips recorded in Tyrode with $[Ca_e]$ 1.1±0.1 mEqv/l		376±9	65.0±1.8	17.7±0.7	2.4±0.1	39.3±1.9	14/7

one the remaining ion variations The difference therefore shows more the normal variation in sodium content, than a difference caused by the change in the potassium concentration in the bath fluid A decrease in the calcium concentration of the bath fluid has a similar effect as for potassium on the calcium content of the strips which decreases from 3 7 to 2 4 mEqv During all the ion variations only the actual ion was influenced The other ions examined remained constant

Spontaneous motility, reactivity to electrical stimulation and membrane potential of animal myometrial preparations are to some extent correlated to the K_i/K_e relationship (Csapo 1956 1959, Goto and Csapo 1959 Jung 1959, Marshall 1962) It is therefore of interest to calculate the K_i/K_e ratio also for human myometrium From the available data such a calculation can be performed by using sodium or chloride space Neither of these gives correct values for the extracellular volume as discussed in the chapter on methods This is, however not necessary for the actual purpose, only the systematical error of the values must be the same This is not the case for sodium space in these experiments as some of the odium which is taken up when the strip is immersed in Tyrode solution is probably bound Even the content of chloride is increased under this condition but the increase is equivalent to the higher content of chloride in Tyrode solution than in plasma For taenia coli Goodford (1964) has shown that the chloride content is correlated linearly to the concentration of chloride in the bath fluid

It must be kept in mind that the K_i and K_e values in the following do not stand for the actual content of potassium intra and extracellularly but that an increase of e g K_i in the actual experiment is accompanied by a corresponding increase of the real K_i content The same is also valid for the other ions

In Table 13 the calculated values for the distribution of ions in the strips, non recorded and after recording in different extracellular ion concentrations are summarized

If the ion values for the non recorded strips and those recorded in Tyrode solution are compared we find that the concentration gradients for both sodium and potassium have decreased *in vitro* This fact suggests that an additional factor *in vivo* not present *in vitro* is necessary to maintain the high transmembrane cation gradients of the intact animal When looking at the high values for internal sodium we must keep in mind that a fraction (14 mEqv) represents the probably bound part of the sodium which has been taken up and is placed intracellularly because of the calculation method

Table 13 Calculated values from Table 12 illustrating the distribution of water and ions in the cell according to the chloride space. Mean values are given ± standard error of mean

Different groups according to the hormonal ions		H_2O_e g/100g	H_2O_i g/100g	Na mEq/100g	Na_i mEq/100g	K mEq/100g	K_i mEq/100g	Cl mEq/100g	Cl_i mEq/100g
	Group 1	2[illegible]7±18	170	38[illegible]±29	38±18	11±01	[illegible]1±1[illegible]	13±03	[illegible]±03
Non-recorded	Group 2+3	81±6	115±7	406±10	21±10	12±01	268±08	14±03	[illegible]1±02
strips	Group 4	[illegible]1±1[illegible]	11[illegible]±16	361±15	16±07	11±01	267±10	13±01	2[illegible]±02
	Group [illegible]	[illegible]71±11	1[illegible]±11	[illegible]99±1[illegible]	32±11	11±01	264±11	13±0[illegible]	25±02
Strips recorded in Tyrode		[illegible]6[illegible]±11	130±19	[illegible]35±10	26[illegible]±21	15±01	161±0[illegible]	10±01	27±02
Strips recorded in Tyrode with [K] 13±01 mEq/l		[illegible]71±17	1[illegible]9±20	371±26	276±29	0.5±0.04	153±09	10±01	29±01
Strips recorded in Tyrode with [K] 8.6±01 mEq/l		240±1[illegible]	1[illegible]9±15	330±11	251±11	20±01	199±03	08±01	31±01
Strips recorded in Tyrode with [K] 11.[illegible]±01 mEq/l		[illegible]47±13	133±15	3[illegible]6±11	[illegible]73±19	26±01	197±07	09±01	12±02
Strips recorded in Tyrode with [K] 15.[illegible]±11 mEq/l		267±13	141±11	371±19	273±21	39±03	202±09	10±01	29±06
Strips recorded in Tyrode with [Ca] 11±01 mEq/l		218±13	13[illegible]±16	312±16	309±26	14±01	161±07	0.30±01	21±01

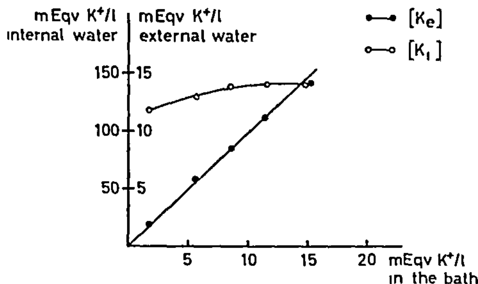


Fig 21 The extracellular and intracellular concentration of potassium at different potassium concentration of the bath The distribution is calculated from chloride space

When the potassium concentration in Tyrode solution is changed the external potassium content changes linearly, and the concentration of potassium per liter external tissue water is the same as in Tyrode solution The internal potassium concentration increases too when the potassium concentration of the bath fluid is increased, but not at all to the same degree (Fig 21) Therefore the relationship between Ki/K decreases e g when the potas ium concentration of the bath fluid is increased The oppo site effect is obtained when K is decreased

DISCUSSION

The ion values for the non recorded strips are comparable to previously described results Cretius (1960) found for corpus uteri 27 9 mEqv of potassium 33 7 mEqv of sodium and 32 0 mEqv of chloride per 100 g dry weight The sodium value is lower but does not significantly differ from that given in the present work Hawkins and Nixon (1958) have estimated sodium potassium calcium magnesium and chloride and used the chloride space for the calculation of the intra and extracellular ion distribution Their results correspond well with those presented here at least for non pregnant myometrium For pregnant myometrium (III—IV month of pregnancy) they found higher potassium values The same conclusion is valid for the results presented by Daniel Hunt and Allen (1960)

Strips recorded in Tyrode solution contain less potassium and more sodium than non recorded strips This phenomenon has not been described before for human myometrium but during the last years for other smooth muscle organs, e g taenia coli (Goodford and Hermansen 1961) and rat aorta (Dawkins and Bohr 1960) They found the same qualitative variation in sodium and potassium movement as described for human myometrium The reason for the change is not definitely known Neither change of temperature nor degree of oxygenation as well as different techniques influenced this cation shift (Goodford and Hermansen 1961) It has also been shown that incubation in plasma fails to maintain the normal ionic equilibrium (Daniel and Robinson 1960)

For all smooth muscle organs tested, the difference in the ion concentration between *in vitro* and *in vivo* is most pronounced immediately after the dissection period After one hour in Tyrode solution some normalization has taken place For taenia coli the ion concentration was then constant for at least 8 hrs (Goodford and Hermansen 1961) There is no reason to believe that human myometrium reacts in another way, especially as the recovery of ions is accompanied by the recovery of the mechanical activity (Barr, Heading and Bohr 1962) It means that during the experimental time, which started about two—three hours after immersion in Tyrode solution, when a steady spontaneous motility was present, the ions have reached equilibrium and remained constant during the whole experimental time

These differences in electrolyte composition advocate the fact that a degree of caution must be exercised in extrapolating from findings *in vitro* to conditions *in vivo* A test *in vitro* is, however, not without interest, as individual characteristics of the smooth muscle, e g mechanical (Berger and Marshall 1961) and electrical (Marshall 1962) performance are retained when the organ is transferred to the isolated bath

In animal myometrial preparations variations in the extracellular potassium concentration are followed by a change in the frequency of the motility For human myometrium similar results are obtained As a fact the frequency of the spontaneous motility of human myometrium increases (Fig 18) in nearly identical way as rat myometrium (Jung 1959), when the extracellular potassium concentration is increased at least within the concentration range 4—16 mEqv/l It is therefore most likely that potassium causes similar changes in the membrane potential of the human myometrium as has been demonstrated for rat uterus i e an increase in the extracellular potassium concentration causes a partial depolarization The ion results obtained for human myometrium suggest this assumption

The relationship K_i/K_e is an expression for the membrane potential Theoretically, calculated by the Nernst equation, the membrane potential varies linearly with the K_e concentration if plotted on a logarithmic scale A high ratio corresponds to a hyperpolarized membrane a low to a depolarized one If we use the K_i and K_e values given for human myometrium the same result is found Direct measurement of the membrane potential on rabbit myometrium indicates however, that the hyperpolarization obtained with low potassium concentration is not as pronounced as could be expected from the calculated values (Goto and Csapo 1959 Marshall 1962) The sensitivity of rabbit myometrium to electrical stimulation at low potassium concentration is also lower than that at normal potassium concentration On rat myometrium Jung (1959) in fact found a decrease of the membrane potential also in absolute figures when the potassium concentration was changed from 5 mEqv/l to 2 mEqv/l As the frequency of the spontaneous motility of human myometrium is further decreased during a similar treatment we can assume that the membrane potential of human myometrium reacts more like that of rabbit myometrium than that of rat myometrium

THE EFFECT OF CHANGES IN THE EXTRA-CELLULAR POTASSIUM AND CALCIUM CONCENTRATIONS ON THE SENSITIVITY AND REACTIVITY PATTERN OF HUMAN MYOMETRIUM TO HSE PG, PGE$_1$ AND PGF$_{1\alpha}$

With the background given in chapter IV the effect of HSE PG, PGE$_1$ and PGF$_{1\alpha}$ has been studied on human myometrium at different extra-cellular potassium and calcium concentrations.

RESULTS

a) *HSE PG*

The material consisted of 64 strips taken from 16 uteri in all phases of the menstrual cycle (Table 14). The potassium concentration was changed in 32 strips from 16 uteri and the calcium concentration in 10 strips from

Table 14. The distribution of the recorded strips where HSE PG was tested in different groups according to the menstrual cycle and to the various potassium and calcium concentration in the organ bath.

Hormonal phase	Groups 1 Early middle proliferative phase	2—3 Late proliferative early secretory phase	4 Middle late secretory phase
No. of strips/uteri in different K^+ milieu	[illegible]/7	21/7	6/2
No. of strips/uteri in different $[Ca^{++}]$ milieu	6/3	8/5	4/2

10 uteri. The remaining 22 strips from 16 uteri were used as controls. The sensitivity and reactivity of the myometrium to HSE PG was first tested on all strips in normal Tyrode solution. Then the extracellular concentration of potassium and calcium was changed in the test bath as outlined in chapter IV. Thereafter the effect of HSE PG was examined and compared

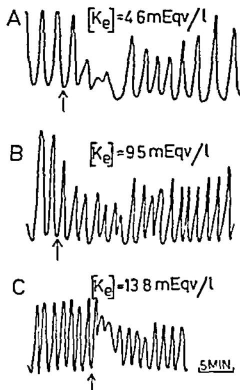


Fig 22 The effect of increasing potassium concentration in the organ bath on the sensitivity and reactivity of human myometrium to HSF PG The uterus is from late proliferative phase At arrows 0 01 U/ml 0 01 U ~ ~ 0 03 μg PGE_1 and 0 004 μg $PGF_{1\alpha}$

with that before in the same bath The stability of the myometrium was checked in the control bath with Tyrode solution throughout the whole experimental time Only such experiments were used where the sensitivity of the myometrium in the control bath remained almost constant

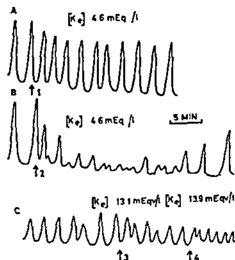


Fig 23 The effect of increasing potassium concentration in the organ bath on the sensitivity and reactivity of human myometrium to HSF PG The uterus is from late proliferative phase 1 = 0 003 U/ml 2 = 3 = 0 03 U/ml 4 = 0 06 U/ml 0 03 U ~ 0 09 μg PGE_1 and 0 01 μg $PGF_{1\alpha}$

The variation in ion concentration gives the same change in the spontaneous motility as described before When the effect of HSF-PG is tested again at different ion concentrations it has changed, while in the control bath it remains almost constant An increase in the potassium concentration up to about 16 mEqv/l decreases the sensitivity of the myometrium to HSF-PG continuously At the highest potassium concentration the effect of a previous clearly inhibiting dose of HSF PG is abolished Here sometimes HSF-PG stimulates the motility (Fig 22) The stimulation is characterized by an increase in tonus In some experiments the degree of sensitivity loss of the myometrium to HSF PG was investigated One of these experiments is illustrated in Fig 23 Here 0 003 units/ml give a slight, and 0 03 units/ml a pronounced inhibition of the spontaneous motility in normal Tyrode solution At a potassium concentration of 13 1 to 13 9 mEqv/l both 0 03 and 0 06 units/ml have no inhibitory effect, instead a slight stimulation is observed When on the other, hand, the potassium concentration is decreased, the effect of repeated doses of HSF PG on the myometrium becomes more and more pronounced (Fig 24) No difference in the reactivity pattern of the myometrium with regard to the menstrual cycle is seen, when the potassium concentration is changed

A decrease in the extracellular calcium concentration which was tested on 10 uteri affects the sensitivity of the myometrium to HSF PG in a more complex way Sometimes (4 uteri) the low calcium concentration (1 0 mEqv/l) obviously increases the sensitivity of the myometrium to HSF PG (Fig 25) The remaining six uteri showed no difference If the material is divided into groups with regard to the phase of the menstrual cycle all the four uteri, where decreased calcium concentration increases the sensitivity of the myometrium, and one of the six, where no difference

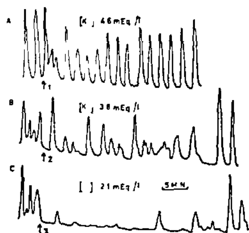


Fig 24 The effect of variations in the extracellular potassium concentration $[K_e]$ on the response of an isolated strips from human myometrium in late proliferative phase At arrows 0 01 U HSF PG 0 01 U ~ 0 03 μg PGE_1 and 0 004 μg $PGF_{1\alpha}$

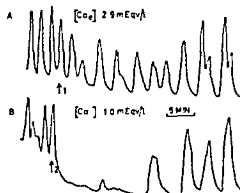


Fig 25 The effect of HSF PG on human myometrium *in vitro* at different calcium concentration The uterus is from early secretory phase 1=2= 0 005 U ml 0 005 U ~ 0 015 μg PGE_1 and 0 002 μg $PGF_{1\alpha}$

is found, belong to group 2 and 3 In these two groups uteri from late proliferative and early secretory phase are placed The remaining five of the six non reactive uteri are from other phases of the menstrual cycle It seems then that a decrease in calcium concentration to 1 0 mEqv/l enhances the inhibitory effect of HSF PG especially around the ovulation

Table 15 The distribution of the recorded strips where PGE_1 and $PGF_{1\alpha}$ were tested in different groups according to the menstrual cycle and to the various potassium and calcium concentration in the organ bath

Hormonal phase	Groups 1 Early middle proliferative phase	2—3 Late proliferative-early secretory phase	4 Middle-late secretory phase
No of strips/uteri in different [K] mileau	15/3	9/2	8/1
No of strips/uteri in different [Ca +] mileau	10/3	6/2	1/1

b) *PGE_1 and $PGF_{1\alpha}$*

The material consisted for PGE_1 of 32 strips from 6 uteri, and for $PGF_{1\alpha}$ of 16 strips from 6 uteri (Table 15) One or two strips from each uterus was used as control The extracellular ion concentration of potassium and calcium was changed in the same manner as for HSF PG

A dose of PGE_1 which inhibited the spontaneous motility was given before and after the change in ion concentration At increasing potassium concentrations the effect of PGE_1 diminishes The decrease in sensitivity of the myometrium is not however of the same magnitude as for HSF-PG

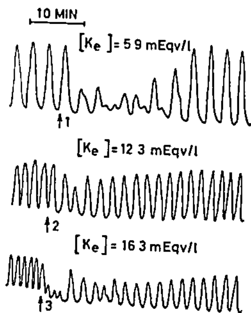


Fig 26 The effect of IGE₁ on an isolated strip from human myometrium in late proliferative phase at different [K] values 1=2=0 01 μg/ml 3=0 03 μg/ml

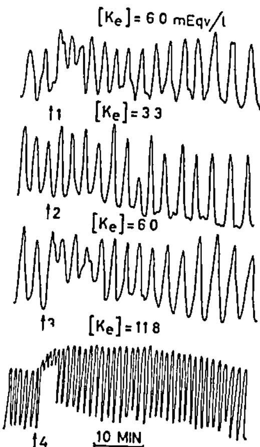


Fig 27 The effect of $PGF_{1\alpha}$ on an isolated strip from human myometrium at different K_e values 1=4=0 5 μg/ml

At the highest concentration of potassium (about 16 mEqv/l) a threefold dose of PGE_1 is almost enough to produce the same degree of inhibition as before in Tyrode solution (Fig 26) A decrease in the potassium concentration pronounces the sensitivity of the myometrium to PGE_1 Even for PGE_1 it seems that a low extracellular calcium concentration (1 mEqv/l) increases the sensitivity of the myometrium at ovulation time

Often a relatively high dose of $PGF_1\alpha$ (0 3—0 5 μg per ml bath fluid) stimulates the motility of the non pregnant myometrium as described in chapter III All 16 strips from the 6 uteri in this part of the investigation showed such a reactivity pattern The effect persists almost unchanged in the control bath throughout the experiments At an increased extracellular potassium concentration the stimulatory effect of $PGF_1\alpha$ is pronounced The stimulation is characterized not only by an increase in tonus but also in frequency A decrease in potassium concentration abolishes the effect of $PGF_1\alpha$ (Fig 27) In no experiments the stimulation changed to inhibition In this material no obvious effect with low calcium concentration on the response of the myometrium to $PGF_1\alpha$ could be observed

DISCUSSION

The effect of HSF PG PGE_1 and $PGF_1\alpha$ on human myometrium is sensitive to the extracellular concentration of potassium A low extracellular calcium concentration also affects the sensitivity of the myometrium to HSF PG and PGE_1 especially at ovulation time

HSF PG contains as described earlier both the three PGE compounds $PGF_1\alpha$ and $PGF_2\alpha$ When the extracellular potassium and calcium concentration is changed the sensitivity and reactivity pattern of the myometrium to HSF PG varies in a way which well corresponds to its content of the PG compounds The more pronounced decrease in sensitivity of the myometrium to HSF PG than to PGE_1 during high potassium concentration can be explained by the interference of the accentuated stimulatory effect of $PGF_1\alpha$ at this condition The assumption that the effect of $PGF_1\alpha$ at high extracellular potassium concentration plays a role for the effect of HSF-PG even on non pregnant myometrium is advocated by the fact that HSF PG now may stimulate the myometrium The stimulation is also similar to that found normally for $PGF_1\alpha$ e g the tonus is increased The change from inhibition to stimulation of the myometrium therefore does not indicate a different reactivity pattern of the myometrium to HSF PG but more reproduces the opposite effect of the PGF and PGE compounds

THE EFFECT OF OVARIAN STEROID HORMONES ON HUMAN MYOMETRIUM

The sensitivity of human myometrium *in vitro* to the PGE-compounds and to HSP PG varies during different phases of the menstrual cycle. This variation may be due to the influence of estrogen and progesterone. Thus the effect of estradiol and progesterone on the sensitivity of human myometrium to PGE_1 *in vitro* was investigated.

In vivo the effect of progesterone described above is accompanied by a firmer binding of calcium in the myometrial cell (Goto and Csapo 1959, Marshall and Csapo 1961). This phenomenon was investigated here for human myometrium in order to both compare the *in vitro* and *in vivo* effects of progesterone, and to study the validity of the same correlation for this tissue. The effect of low calcium concentration in the organ bath on the calcium content of pregnant myometrium (high progesterone influence) and non pregnant myometrium (low progesterone influence) was compared as well as the effect of progesterone added to the bath fluid.

SPECIAL METHODS

PGE_1 was used as a representative for the prostaglandins. Progesterone and estradiol 17β were used (kindly supplied by AB Pharmacia). The compounds were dissolved in absolute ethyl alcohol, 1 mg per ml, and then added to the Tyrode solution until a final concentration of 10 µg/ml was reached. The hormones were added to the bath fluid in the same manner as was potassium. The sensitivity of the myometrium to PGF_1 was first tested for all strips in Tyrode solution. Then progesterone and/or estradiol were added and the effect of PGE_1 again examined. Each strip was used as its own control. The stability of the sensitivity of the myometrium to PGE_1 was determined in one or two control baths with Tyrode solution throughout the whole experimental time. At the end of the experiments the content of sodium, potassium and calcium in the strips were examined.

Initially the material tested consisted of 34 uteri. In order to accurately evaluate the results only those experiments were used where the uterus in the control bath kept its sensitivity to PGF_1 fairly constant during the whole experiment. When progesterone and/or estradiol were administered

to the bath fluid the spontaneous motility often continuously decreased until it finally stopped. As PGI_1 also inhibits the motility it had to be given before the motility stopped. In fact only those experiments were used where the motility recovered after the effect of PGI_1 had disappeared. This is possible as the effect of the hormones develops slowly. After this selection 82 registrated strips from 17 uteri remained. Their distribution between different phases of the menstrual cycle and various hormonal treatments is given in Table 16.

Table 16. The distribution of strips and uteri with regard to different phases of the menstrual cycle and to different hormonal treatment

Treatment	Phase in the menstrual cycle	Early middle proliferative phase	Late proliferative early secretory phase	Middle late secretory phase
Controls in Tyrode	Strips	9	9	[illegible]
	Uteri	6	7	4
Tyrode + 10 μg progesterone	Strips	8	9	[illegible]
	Uteri	5	7	3
Tyrode + 10 μg oestrodiol	Strips	7	8	4
	Uteri	5	6	3
Tyrode + 10 μg estradiol and progesterone	Strips	6	9	3
	Uteri	4	6	2

In the calcium experiments the calcium concentration in the organ bath was lowered to about 1.0 mEq/l. To half of these baths 10 μg/ml progesterone was also added. In the control baths the calcium concentration was the normal one, 3.0 mEq/l. The content of calcium as well as of potassium and sodium was determined at the end of the experiment. The difference in content of calcium between the strip in the experimental bath both with and without progesterone, and the strip in the control bath was examined. Seven non pregnant and 5 pregnant uteri were used in this study.

RESULTS

a. *The effect of estradiol and progesterone on the sensitivity of the myometrium to PGE_1*

The sensitivity of the myometrium to PGE_1 *in vitro* can be affected by the hormones investigated. The effect is most pronounced at ovulation time

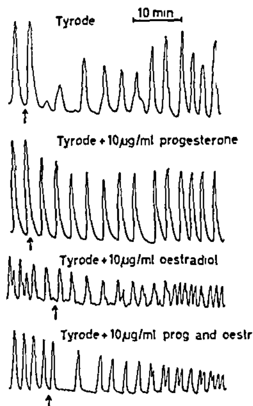


Fig 28 The effect of PGE_1 on human myometrium *in vitro* in Tyrode and in Tyrode with estradiol and/or progesterone The uterus is from late proliferative phase At arrows 0 03 µg PGE_1/ml

when the sensitivity of the myometrium is highest Here progesterone and estradiol together with progesterone decrease the sensitivity of the myometrium to PGE_1, i e decrease the inhibition evoked by PGE_1 (Fig 28 This reactivity pattern is observed in 5 uteri In no case the steroid hormones increase the sensitivity of the myometrial strips to PGE_1

During early and middle proliferative phase estradiol increases the sensitivity of the myometrium to PGE_1 in only one uteri of the five investigated In the other 4 uteri the inhibitory effect caused by PGE_1 was unaffected or *slightly diminished by estradiol In this phase of the menstrual cycle* progesterone sometimes increases the sensitivity of the myometrium to PGE_1 Two of the 5 uteri in this phase showed this reactivity pattern (Fig 29) On the remaining 3 uteri no effect was observed

In the last group containing strips from uteri in middle and late secretory phase no effect is obtained either with estradiol or progesterone

No statistical differences are found between the ion content, either during the various phases of the menstrual cycle, or under different hormonal treatment

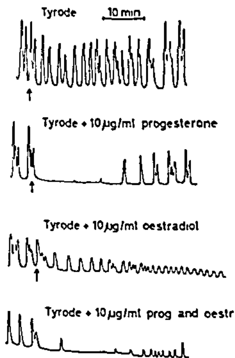


Fig 29 The effect of PGE_1 on human myometrium *in vitro* in Tyrode and in Tyrode with estradiol and or progesterone The uterus is from early middle proliferative phase At arrows 0 1 µg PGE_1/ml

b *The effect of low calcium concentration in the organ bath*

The differences in calcium content of the strips in the experimental bath, with low calcium concentration together with or without 10 µg/ml progesterone, and of those in the control bath, are given in Table 17 The concentration of sodium, potassium and chloride not given in the table is the same in the experimental and in the control strips When non pregnant myometrium is bathed in Tyrode solution with low calcium concentration ($1\,06 \pm 0\,08$ mEqv/l) the calcium content of the myometrium decreased by $4\,04 \pm 0\,20$ mEqv/1000 g wet weight in comparison to the control strip from the same uterus in normal Tyrode solution When progesterone is added to the bath fluid the decrease is slightly less The difference is not statistical significant ($0\,5 > P > 0\,2$) If pregnant myometrium is used instead where a higher endogenous progesterone influence is present than for the non pregnant myometrium the change in calcium content between the strip in the control bath and that in the experimental bath is lower ($3\,04 \pm 0\,10$ mEqv/1000 g wet weight) The difference between the pregnant and non pregnant myometrium is statistically significant ($P < 0\,001$) The difference is probably higher than indicated in the Table as the calcium concentration in the bath fluid for the non

Table 17 The difference in calcium content of human pregnant and nonpregnant myometrium in normal Tyrode in Tyrode with low calcium content and in Tyrode with low calcium content together with progesterone The values given are expressed as mEqv/1000 g wet weight

Non pregnant myometrium		Pregnant myometrium	
Difference in Ca content of strips in normal Tyrode (Ca 3 96±0 02 mEqv/l)		Difference in Ca⁺⁺ content of strips in normal Tyrode (Ca⁺⁺ 4 20±0 06 mEqv/l)	
and Tyrode with low Ca⁺⁺ conc (Ca 1 06±0 08)	and Tyrode with low Ca⁺⁺ conc (1 03±0 07) and 10 μg/ml prog	and Tyrode with low Ca⁺⁺ conc (1 10±0 05 mEqv/l)	and Tyrode with low Ca⁺⁺ conc (1 20±0 013 mEqv/l) and 10 μg/ml prog
4 1	4 7	3 1	3 9
4 7	4 2	2 9	3 1
4 8	4 6	3 0	2 3
3 8	3 3	2 8	2 1
3 3	3 3	3 4	3 4
4 0	2 3		
3 6	3 9		
4 04±0 20	3 76±0 32	3 04±0 10	2 96±0 34

pregnant myometrium is decreased by 2 90 mEqv/l, and for the pregnant myometrium by 3 1 mEqv/l Progesterone added to the experimental bath has no effect on the release of calcium from the pregnant myometrium

DISCUSSION

The dose of progesterone and estradiol used (10 μg/ml) is the usual one employed by other authors and is high enough to influence electrical and mechanical activity as well as the sensitivity of the myometrium *in vitro* (Kumar Barnes and Scott 1961, Kuriyama 1961, Marshall and Csapo 1961) It is higher than the concentration found in human blood both during different phases of the menstrual cycle (0 6—2 1 μg/100 ml in the post ovulatory phase, Woolever 1963) and during pregnancy (5—25 μg/100 ml Zander 1961) Even umbilical venous blood contains less, about 45—60 μg/100 ml (Sommerville *et al* 1963) The higher dose of pro gesterone needed *in vitro* may be taken as an indication that progesterone *does not take part in the* regulation of the activity and sensitivity of the myometrium *in vivo* However, the hormone concentration *in vivo* may not be uniformly distributed in the myometrial tissue If the target organ of progesterone is the excitable membrane it may be possible, that the major

part of the progesterone is taken up in the membrane and gives a much higher concentration locally Values as high as 10 μg/g have been calculated (Csapo 1961)

An effect of a high endogenous progesterone influence such as that during pregnancy and that following progesterone treatment of the estrogen dominated animal *in vivo* is to hyperpolarize the membrane, to reduce the frequency and amplitude of both the spontaneous action potentials and of the motility and to depress the sensitivity of the myometrium to agents such as oxytocin (Marshall 1959, Csapo 1959, 1961, Kuriyama 1961) It is then of interest to recall that during pregnancy and during middle late secretory phase when the progesterone production is relatively high the sensitivity of human myometrium to the PGE compounds is low

Progesterone treatment of myometrium *in vitro* gives in some respects the same effects as described *in vivo* (Csapo 1961, Marshall and Csapo 1961, Kuriyama 1961) However, differences are also present First the *in vitro* effect of progesterone develops more instantaneously whereas the *in vivo* effect appears following several hours delay After *in vivo* treatment the uterus shows a characteristic inverse relation between tension and frequency of stimulation (the negative staircase phenomenon) which is absent after *in vitro* administration of progesterone At ovulation time progesterone administered *in vitro* decreases the sensitivity of human myometrium to PGE_1 This result may indicate qualified by differences in effect of progesterone *in vivo* and *in vitro* that the change in sensitivity of human myometrium to the PGE compounds during different phases of the menstrual cycle found is due at least in part to variations in the progesterone production

Estradiol fails to increase the sensitivity of human myometrium to PGE_1 *in vitro* An opposite effect could have been expected with regard to the effect of estradiol *in vivo* where the hormone increases the sensitivity of at least animal myometrial preparations (Marshall 1959, 1962 Jung 1961 b) The effect is, however in accordance with the finding that estradiol treatment *in vitro* in contrast to *in vivo* decreases the sensitivity of myometrium to electrical stimulation in the same way as progesterone (Csapo 1961) and the result therefore does not exclude the possibility that even estradiol is of importance for the sensitivity of human myometrium to the PGE compounds

An uterus during progesterone influence binds calcium more firmly in the cell at the same time the sensitivity of the myometrium is low The two results have been correlated and the proposal suggested that the sensitivity of the myometrium is dependent on the degree of binding of calcium

(Goto and Csapo 1959, Marshall and Csapo 1961) The results presented here indicate that a similar mechanism may be possible even for human myometrium

Progesterone treatment of human myometrium *in vitro* is not as effective on the calcium release as a high progesterone influence *in vivo* This condition may serve as an explanation for the less pronounced effect given by progesterone *in vitro* on the sensitivity of human myometrium to PGE_1 if compared with the variation in sensitivity normally found during different phases of the menstrual cycle

GENERAL DISCUSSION

Euler (1934, 1935) and Goldblatt (1933, 1935) independently demonstrated the presence of blood pressure depressing and smooth muscle stimulating material in extracts of human seminal plasma and of vesicular gland of sheep Euler (1936) named the factor provisionally prostaglandin and showed that it was lipid soluble and had acidic properties

Starting with extracts of vesicular gland of sheep Bergstrom and Sjovall (1960 a, b) first isolated two prostaglandin compounds PGE_1 and $PGF_{1\alpha}$ with the chemical formulas $C_{20}H_{34}O_5$ and $C_{20}H_{36}O_5$ respectively Further investigations showed the presence of two additional compounds PGE and PGE_3 (Bergstrom *et al* 1962 b) Two other PG compounds $PGF_{2\alpha}$ and $PGF_{3\alpha}$ have also been found in extracts of biological tissues (c f Samuelsson 1963 b 1964) The chemical structure of all these compound have been clarified (Bergstrom *et al* 1962 b c ,d, Samuelsson 1963 a) (See Fig 1) PGE_1 is 11α 15-dihydroxy 9 keto-prost 13 enoic acid PGE_2 differs from PGE_1 by an Δ^5 double bond PGE_3 has a third double bond between C 17 and C 18 The PGF compounds have a hydroxy group at C 9 instead of the ketogroup found in the PGE-compounds

The different prostaglandin compounds are present in many organs (Euler 1934 1935 Goldblatt 1933, 1935, Euler and Hammarstrom 1937, Eliasson 1959, Bergstrom and Sjovall 1960 a b, Bergstrom *et al* 1962 a b Bergstrom and Samuelsson 1963, Samuelsson 1963 b Eglington *et al* 1963 Ånggard and Samuelsson 1963 1964, Ånggard 1964, Horton and Thompson 1964) The highest amount is found however, in human seminal plasma and sheep vesicular gland (Eliasson 1959 Bergstrom *et al* 1962 b d, Samuelsson 1963 b, Horton and Thompson 1964)

Different results presented strongly suggest that the prostaglandins in human and sheep semen are formed in the seminal vesicles and in the vesicular glands respectively (Eliasson 1959 Bergstrom *et al* *1964* a, b Dorp *et al* 1964 a b)

In human seminal plasma 5 prostaglandin compounds are present PGE_1 PGE_2 PGE_3 $PGF_{1\alpha}$ and $PGF_{2\alpha}$ (Bergstrom and Samuelsson 1963 Samuelsson 1963 b) The concentration of prostaglandin in human semen has been estimated in different ways, either by measuring the smooth muscle activity of semen (Asplund 1947 a Eliasson 1959 Hawkins and

Labrum 1961, Horton and Thomson 1964) or by a chemical method devised by Bygdeman and Samuelsson (1964) (See chapter II) With the latter method it is found that a pool of human semen, obtained from a sterility clinic, contains 15 5 μg PGE_1, 12 8 μg PGE_2 2 5 μg PGE_3, 2 0 μg $PGF_{1\alpha}$ and 2 2 μg $PGF_{2\alpha}$ per ml The values are mean values (See Table 1) By means of the different activity of the PGE- and PGF compounds on isolated rabbit jejunum they can be transferred to PGE equivalents (See chapter II) The semen pool then contains about 100 μg of PGE_1 equivalents per ml Earlier the smooth muscle activity of human semen was expressed in units in comparison to a standard preparation of prostaglandin On rabbit jejunum 1 unit corresponds to 7 5 μg of PGE_1 The same type of semen as described above contains according to Eliasson (1959) about 12 units ~ 90 μg of PGE_1 per ml and according to Asplund (1947 a) about 9 units ~ 70 μg of PGE_1 per ml Semen from men with documentated normal fertility definitely contains more prostaglandin determined both with the chemical method (Bygdeman and Samuelsson, to be published) and with the biological method (Eliasson 1959, Horton and Thompson 1964) Single semen samples from infertile men sometimes, on the other hand, have hardly any smooth muscle activity (Asplund 1947 a, Eliasson 1959, Horton and Thompson 1964)

All the PG-compounds and crude prostaglandin extract from human semen and vesicular gland of sheep strongly stimulate almost all smooth muscle organs tested (Bergstrom *et al* 1959 b, Eliasson 1959, Bergstrom and Euler 1963, Horton and Main 1963, Änggard and Bergstrom 1963) One exception is in most cases, the Fallopian tube from rabbit and human where prostaglandin and the PGE-compounds inhibit the spontaneous motility (Asplund 1947 b, Horton and Main 1963 Sandberg *et al* 1963 a, b) The most proximal part, however, is stimulated by prostaglandin and PGE_1 (Sandberg *et al* 1963 a, b) Also HSF-PG applied intravaginal to infertile women, sometimes increases the tonus of the Fallopian tube measured by Rubin s test (Eliasson and Posse 1964) Blood pressure of rabbit cat, dog and human are lowered, most pronounced by the PGE compounds and crude prostaglandin but also by the PGF-compounds (Euler 1936 1939 Eliasson 1959, Änggard and Bergström 1963, Bergstrom and Euler 1963)

The results presented in this work show that the spontaneous motility of human myometrium is inhibited by the PGE but stimulated by higher doses of the PGF-compounds present in human semen The effect of the PGE-compounds corresponds to that found for HSF PG, a crude extract of the smooth muscle activity of human semen

On human myometrium all PGE compounds have almost the same activity, indicating that the number of double bonds appears to have little importance for their activity on this organ This is not the case however for other smooth muscle organs tested, where usually F_2 is the most potent and F_3 the least active of these substances (Bergstrom and Euler 1963, Horton and Main 1963) The keto group however, seems to be essential for the inhibitory effect, since PGF$_1\alpha$ and PGF$_2\alpha$ have a tendency to stimulate the myometrium instead

In human semen and in HSF PG it is the PGE compounds which are essential for the normal inhibitory response This fact is of importance since it limits the results obtained in previous studies of the content of prostaglandins in human semen with regard to their effect on the myometrium The concentration in the samples usually was measured on rabbit isolated jejunum (Asplund 1947 a Eliasson 1959, Hawkins and Labrum 1961, Horton and Thompson 1964) As all PG compounds strongly stimulate this preparation such a method gives the total amount of the compounds Variations in the concentration of the individual PG compounds are not detected This means for instance, that single semen samples containing an abnormally high concentration of PGF compounds may give a normal result on isolated rabbit jejunum but no inhibitory effect on human myometrium That a variation in the concentration of the PG compounds is present and of importance is indicated by the results obtained by determinations of the various prostaglandins in single semen samples from infertile men using the chemical method described by Bygdeman and Samuelsson (1964) In some cases only a few µg of the PGE compounds per ml semen were found (Bygdeman and Samuelsson to be published)

Eliasson (1959) found in young fertile women that prostaglandin deposited in the vagina at ovulation time stimulated the motility of the myometrium in the corpus part The stimulation changed to inhibition in some cases after 10—20 min During coitus oxytocin is normally liberated (Cambell and Petersen 1953 Friberg 1953 Harris and Pickles 1953, Pickles 1953) If this condition is imitated in the experiments by infusion of post pituitary hormones at the same time as prostaglandin was deposited in the vagina a marked inhibition is found within 2—5 min probably due to the effect of oxytocin which increases the sensitivity of myometrium to both intrinsic and external stimulation (Csapo 1954) The inhibitory effect of prostaglandin is then probably the normal one at coitus Eliasson suggested on the basis of the comparative physiology and reproduction of various animals and man and of experimental data obtained with prosta

glandin, that if it is 'an increase in tonus of the uterus at coitus and orgasm followed by a marked decrease in tonus evoked by prostaglandin (with the present knowledge the PGE compounds) this would seem likely to facilitate a passive sperm migration from the semen pool around the portio into the uterine cavity Eliasson also suggested that this is the physiological function of prostaglandin with regard to conception Naturally the effect of prostaglandin is only one factor among many of importance for normal fertility In some cases however it may play an essential role Couples, where the husband had oligospermia and no contraindication to conception was detected in the wife had a greater possibility for conception if the smooth muscle activity of the semen was normal, than if the activity was low (Hawkins and Labrum 1961) In men with a normal number of sperm no correlation was found between conception and prostaglandin content In this investigation as pointed out before the prostaglandin content was estimated with regard to the total smooth muscle activity of the semen It might be expected that an analysis of the individual PG-compounds will give more information in this field

It is a well known fact that sensitivity and reactivity of myometrial preparations from many animals vary during the estrus cycle The sensitivity is most pronounced during estrus (Bozler 1938 1941, 1948 Csapo 1959 1961 Kuriyama 1961, Marshall 1962) If a similar variation is investigated on human myometrium, it is natural to divide the uteri in at least two groups with regard to the menstrual cycle One group containing uteri from ovulation time corresponding to the estrus phase, and the second uteri from other phases of the cycle

Such a division was used for investigation of the effect of different prostaglandin compounds on human myometrium It was also shown that the sensitivity of the myometrium was most pronounced in the first group If on the other hand, the whole proliferative and the whole secretory phase are compared, no difference in sensitivity was found The most sensitive uteri those from the ovulation time, are then equally distributed in the two groups It is also natural that the sensitivity of the myometrium is most pronounced at ovulation time with regard to the proposed physiological role of prostaglandin to enhance the passive transport of the sperms in the female genital tract

It has been postulated that the variation in sensitivity of animal myometrial preparations is due to the endogenous production of estradiol and progesterone The assumption is mostly based on the fact that the hormones administered to animals cause the same variation in reactivity pattern of the uteri as normally found during the different hormonal

phases Even *in vitro* treatment with the hormones often changes the sensitivity in a way similar to that *in vivo* (Bozler 1938, Goto and Csapo 1959, Marshall and Csapo 1961, Kuriyama 1961, Jung 1961 a b)

Estrogen administered to spayed animal increases the spontaneous activity and reactivity of the myometrium to the same degree as found during the normal estrus phase The effect is probably due to an increase of the actinomyosin content (Cretius 1957, Csapo 1949) and to an increase of the membrane potential over a critical value where action potentials can be produced (*c f* Marshall 1962) If progesterone is given after previous treatment with estradiol the spontaneous motility becomes irregular and both sensitivity and conduction of impulses are depressed (Csapo 1959, Goto and Csapo 1959, Kuriyama 1961, Marshall 1959) Csapo introduced the term progesterone block for this phenomenon

Application of the results obtained in animals may give a basis for an explanation of the different sensitivity of human myometrium to the PGE compounds and to HSF PG during different phases of the menstrual cycle and during pregnancy

During middle late secretory phase and during pregnancy, when the progesterone production is most pronounced (*c f* Carey 1963) the sensitivity of the myometrium to the PGE compounds and to HSF PG is lower than during ovulation time, when, on the other hand, the estrogen production reaches a peak (*c f* Brown 1963) The correlation is further emphasized by the results obtained *in vitro* where progesterone decreases the sensitivity of the myometrium to PGF_1 if administered to uteri from ovulation time

The effect of progesterone on the sensitivity of the myometrium was first proposed to be mediated by a decrease in the intracellular potassium concentration and an increase in the intracellular sodium concentration (Horwath 1954) Later investigations on animal myometrial preparations including membrane potential measurement have not given further support to this assumption but instead suggested the key role of calcium Calcium, which is a membrane stabilizer is more firmly bound in the cell during progesterone influence This phenomenon is the eventual reason for the depressing effect of progesterone for the progesterone block (Goto and Csapo 1959 Marshall and Csapo 1961)

The results presented in this work indicate that the same mechanism may also be valid for human myometrium In human myometrium the calcium content is more resistent to exposure to low extracellular calcium concentrations if the endogenous progesterone production is high (pregnant myometrium) than if it is low (non pregnant myometrium)

The effect of the variations in the extracellular potassium concentration on the sensitivity of the non pregnant myometrium to the PG compounds tested and to HSF PG, is pronounced (chapter V) An increase in the potassium concentration decreases the inhibitory effect of the PGI compounds and of HSF-PG On the other hand, the stimulatory effect of the PGI compounds is enhanced The variation in sensitivity of the myometrium is so marked that it well could explain the changes in sensitivity found during the different phases of the menstrual cycle Such an explanation, however, is not in accordance with the ion results presented No variation in the total *ion content* was found, either in myometrial strips taken from the myometrium immediately after the operation, or in the strips regis rated in Tyrode solution Also Daniel, Hunt and Allen (1960) found no differences in ion content of human myometrium during various phases of the menstrual cycle Probably no changes even in the intra extra cellular distribution take place This proposal is based on the finding that the ion distribution in the myometrium calculated by the chloride space showed no difference during the various phases of the menstrual cycle However, the chloride space is not equal to the extra cellular volume and it may be possible that a redistribution of ions takes place If this change is the same for chloride as for the other ions, the above described result will be obtained Preliminary determinations of the extracellular space of human myometrium by inulin, however, give no support for the second possibility (Bygdeman, unpublished observation) Even in rabbit no difference in the extracellular space, measured by inulin is found if progesterone and estrogen-dominated uteri are compared (Kao 1961)

Progesterone administered *in vitro* was also without effect on the ion content of the myometrium in normal Tyrode solution Eventually high local influence of progesterone might, however, change the concentration of potassium (Barnes and Kumar 1961)

The importance of calcium for the variation in sensitivity of the myometrium during the different phases of the menstrual cycle is further advocated by the fact that a decrease to one third of the normal concentration in Tyrode solution also increases the sensitivity of the myometrium to HSF PG and to the PGF compounds, most pronounced at the ovulation time

Of interest are the results of Clegg Hopkinsson and Pickles (1963), who found that myometrium of guinea pig was most sensitive to menstrual fluid extract (component A) at low Ca concentrations (0 7 mEqv/l)

Component A has later been shown to contain PGF and $PGF_{2\alpha}$ (Eglington *et al* 1963)

The effect of isoxsuprine which also inhibits the spontaneous motility of human myometrium has been suggested to be due to an activation of the adrenergic beta receptor system (Lish Hillyard and Dungan 1960) It is possible that the PGF compounds which also inhibit human myometrium act in a similar way

However some results obtained with isoxsuprine in comparison with those of the PGE compounds, indirectly indicate that this is not the case The effect of isoxsuprine is quite different from that of the PCE compounds The latency period is longer and isoxsuprine seems to prolong the first phase of the relaxation a result not found for the PGF compounds The effect of isoxsuprine is not influenced by variations in the extracellular potassium concentration as is that of PGE_1 The ID_{50} dose for isoxsuprine is for human myometrium 25 μg/ml bath fluid (Bygdeman and Eliasson 1963 e) The corresponding dose for PGE_1 at ovulation time is 0 01—0 05 μg/ml bath fluid

Preliminary results obtained on human myometrium with a beta receptor blocking agent (ICI 45 520) also indicate that the effect of prostaglandin is not influenced by this type of substances (Eliasson, personal communication) Therefore the effect of the PGE-compounds is probably not mediated by an activation of the beta adrenergic receptors

SUMMARY

The effect of different prostaglandins present in human semen, PGE$_1$, PGE$_2$, PGE$_3$, PGF$_{1\alpha}$, PGF$_{2\alpha}$, and a crude extract of human semen, HSF PG, has been investigated and compared on human myometrium *in vitro*

The concentration of the prostaglandins in HSF PG is determined with a new chemical method HSF-PG contains PGE$_1$ 12 3 μg, PGE$_2$ 6 6 μg, PGE$_3$ 1 2 μg, PGF$_{1\alpha}$ 1 3μg and PGF$_{2\alpha}$ 1 5 μg per ml semen The smooth muscle activity of HSF-PG is also estimated and a comparison between the content of prostaglandins expressed in units and in μg is performed

All the PGE compounds inhibit the spontaneous motility of human non pregnant myometrium *in vitro* The ID$_{50}$ dose, e g the dose which decreases the amplitude of the contractions to 50% during at least 10 min, is at ovulation time 0 01—0 05 μg/ml bath fluid The PGF compounds, in higher doses, 0 3—0 5 μg/ml, on the contrary, often stimulate the motility

In most cases the effect of HSF PG is similar to that obtained with the PGE compounds At ovulation time the ID$_{50}$ dose of HSF PG is 0 001—0 01 U/ml The effect of 1 unit corresponds to that obtained with 3 μg of PGE$_1$ This relationship well corresponds to the estimated concentration of the prostaglandins in HSF PG

The PGE compounds also inhibit the motility of pregnant myometrium, although the ID$_{50}$ dose, 0 03—0 1 μg/ml, is higher than that found for non pregnant myometrium The stimulatory effect of the PGF compounds tested is more pronounced on pregnant myometrium A stimulation is often seen with 0 01—0 03 μg/ml Even HSF PG stimulates the major number of pregnant uteri tested This effect is probably due to the higher sensitivity of the myometrium to the PGF compounds and does not indicate a different reactivity pattern of the pregnant myometrium

The sensitivity of non pregnant myometrium to the different prostaglandins and to HSF PG is dependent on the extracellular concentration of potassium and calcium An increased potassium concentration makes the myometrium less sensitive to the PGE compounds and to HSF PG

but enhances the effect of the PGF compounds A decrease in the calcium concentration to ⅓ of the normal one increases the sensitivity of the myometrium to HSF PG and to PGE_1 predominantly at the ovulation time The variation in ion concentration of the Tyrode solution is correlated with changes in the ion content and the distribution of the ions in the myometrium The significance of these variations is discussed

The sensitivity of the non pregnant myometrium to the PGE compounds and to HSF PG *in vitro* varies significantly during different phases of the menstrual cycle The sensitivity of the myometrium is most pronounced at ovulation time The reason for this variation is not clear Progesterone decreases, however, the sensitivity of the myometrium to PGE_1 if administered to uteri from ovulation time. A similar decrease is found when a uterus from ovulation time and one from middle late secretory phase is compared It is suggested that the variation in sensitivity might in part be due to the influence of the ovarian hormones on the myometrium

The variation in sensitivity of the myometrium during changes in the extracellular potassium concentration is so marked that it well can explain the variation found in sensitivity during the different phases of the menstrual cycle Such a mechanism for the hormone influence is however, not in accordance with the finding that the ion content and the distribution of ions in the myometrium during the menstrual cycle is constant On the other hand some results obtained suggest that calcium in this respect may be of importance

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THE INSECT CORNEAL NIPPLE ARRAY

A biological, broad-band impedance transformer that acts as an antireflection coating

BY

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CONTENTS

GENERAL INTRODUCTION

When energy passes from one material to another in which its propagation velocity is different, some is reflected at the boundary and the balance is transmitted in the second material. This is true for both electromagnetic and pressure (sound) waves. It is the purpose of impedance matching devices to minimize reflections at such boundaries and hence to maximize the transmission.

Such man made devices are so well known and of such ancient origin they scarcely need reiteration. A most familiar one is the megaphone (or simply cupped hands as its primitive aspect), which serves to match the radiation impedance of the mouth to that of air by means of providing a gradual area transition between the two. And when this acoustic horn is applied to the ear rather than the mouth, it serves as an admirable hearing aid by matching the impedance of air to that of the middle ear.

In the middle ear we find a good example of the living organism's effort to reduce reflection from an air fluid interface. This anti reflection device is, therefore, a natural impedance transformer: the delicate anatomical structures of the middle ear serve for a wide range of wavelengths to match the impedance of air to that of the fluid filled inner ear where the receptor "hair" cells are located. They are so successful in their function that almost the maximum transfer of energy that is theoretically possible is achieved, accounting for the truly incredible sensitivity of the ear.

Biological impedance transformers are thus not unknown, and the subject matter of this paper deals with another example: the insect corneal nipple array. The anatomical and functional properties of this array as elucidated by our experiments are described in the following pages.

PART I

ANATOMY OF
THE CORNEAL NIPPLE ARRAY

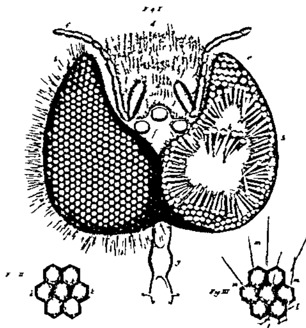


Fig 1 Head of the honey bee showing structure of the compound eyes according to Swammerdam, Bijbel der Natuure Leyden 1737

A LIGHT MICROSCOPY

The compound eyes of arthropods are composed of numerous separate small eyes called ommatidia each having its own sensory and dioptric system (Fig 1) The number of ommatidia in different arthropod compound eyes varies from a single one (not to be confused with the ocellus) up to about 30,000 in some insects The dioptric apparatus usually consists of a cornea and underlying crystalline cone The cornea is a transparent extension of the general chitinous cuticle that is often bi convex or plano-convex in shape The outer surfaces of these corneal lenslets are the facets of the compound eye

Our knowledge of the structure of the arthropod cornea is derived for the most part, from observations made with the light microscope These investigations suggest that the corneal front surfaces are always completely smooth This is illustrated by Fig 2 which shows photomicrographs of a fly (A) and a night moth (B) cornea (with underlying crystalline cones)



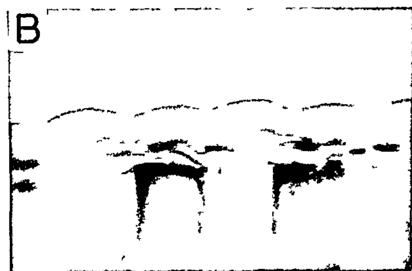


Fig 2 Photomicrographs of the cornea of *Musca* (A) and *Prolenia* (B) sectioned normal to corneal surface × 10,0

sectioned normal to a plane tangent to the corneal surface The micrographs were made using the oil immersion objective, and in these pictures the front surface of the corneal facets appear to be smooth Such pictures are typical of the arthropod compound eye cornea regardless of the class the animal is taken from

B ELECTRON MICROSCOPY

When viewed by means of the electron microscope, too the front surface of the eyes of numerous insects appears to be completely smooth We found this to be so for *Apis* (bee), *Bombus* (bumblebee) [Hymenoptera] *Coccinella* (ladybird) [Coleoptera] and the compound eye of *Limulus* [Arachnoidea] The appearance of such corneas in electron micrographs is illustrated by Fig 3 which depicts a bee cornea

The front surface of the corneas of certain other insects (Fig 4), however is seen with the electron microscope to be completely covered with conical protuberances that we have called corneal nipples (Bernhard and Miller, 1962) In such insects these nipples are present only on the corneal chitin We have not observed them on extracorneal chitin, an example of which is depicted in Fig 5 The insects in which we have found corneal nipples are *Vanessa* (butterfly), *Prodenia Cerapteryx Sphynx Endromis* (moths) [Lepidoptera] *Myrmeleon* (netfly) [Neuroptera], *Phryganea* (caddis fly) [Trichoptera], and *Aedes Culex*, and *Anopholes* (mosquitos) [Diptera] The nipples have also been found in the developing eye of *Aedes* (White R H Purdue Univ , personal communication)

The appearance of the shape of the nipples is bound to be dependent on the plane of section Thus in Fig 6 the nipples on the middle facet are cut obliquely and at different levels on the nipples as the section passes through different nipple rows (see also Fig 7) On the side facets of Fig 6 and the facets depicted on Figs 4, 8 9 and 10 the plane of the section passes more nearly through the altitude of some of the nipples The appearance of the spacing of the nipples is likewise dependent on the plane of the section On these same sections the variety of spacings is due to the angles the planes of the sections made with the nipple rows Fig 11 shows a micrograph of a section made tangent to the corneal facet and slightly above the surface This corfirms the fact that the nipples are congruent cones arranged in a more or less perfect hexagonal array

Although the regularity of both the nipple shape and spacing is evident it is difficult to form a true three-dimensional reconstruction of the nipple pattern For all of the insects in which corneal nipples were found, both their average height and center to-center distance is estimated to be about 200 mμ but it is not certain as to whether or not the nipples are placed as close together to each other as possible If they are such an arrangement of the nipples will only rarely be visualized in the electron microscope pictures of random sectioned samples Since there is such a close arrangement within certain regions of the sections at the nipples bases (Figs 4 8 9 and 10) we conclude that the nipples might very well be located close side by side

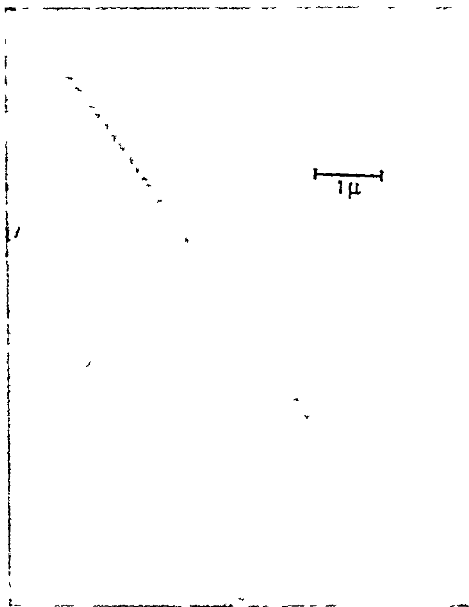


Fig 3 Electron micrograph of a bee cornea sectioned normal to corneal facet surface.

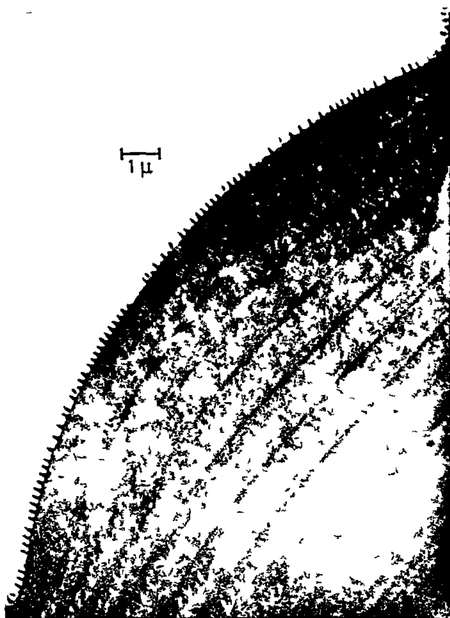


Fig 4 Electron micrograph of a butterfly cornea *(Rrhacia)* sectioned normal to corneal facet surface



Fig 5 Electron micrograph of extracorneal chitin from a night moth *(Prodenia eridania)* sectioned normal to body surface

The structure of the housefly, *Musca domestica* [Diptera], cornea presents a variant of the nipple array Fig 12 shows the cornea of housefly in an electron micrograph It is seen that the front surface of the cornea is smooth It is also noted that in contrast to the smooth front surface of the bee, that of the fly has a rather dense cortical layer Thinner sections of the cornea reveal that the cortex actually contains a nippled substructure (Fig 13) But in this case the spaces between the nipples are completely filled in with corneal material with the result that the front surface of the cornea does not show the nippled structure

To summarize, our present knowledge indicates that the front surfaces of arthropod corneas are either smooth or completely covered with the nipple array The smooth surfaced types may or may not have a substructure of nipples

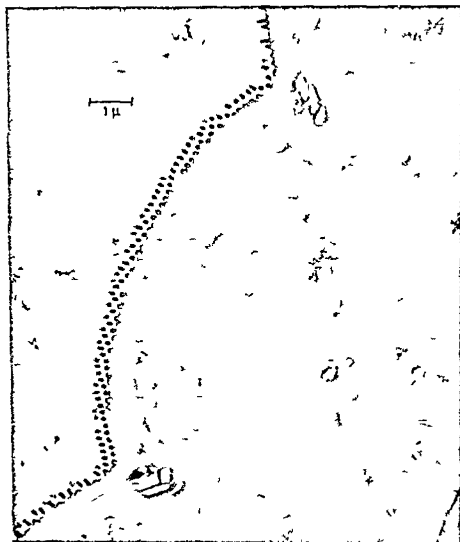


Fig 6 Electron micrograph of a night moth cornea *(Prodenia eridiana)* sectioned oblique to the corneal nipples of the middle facet

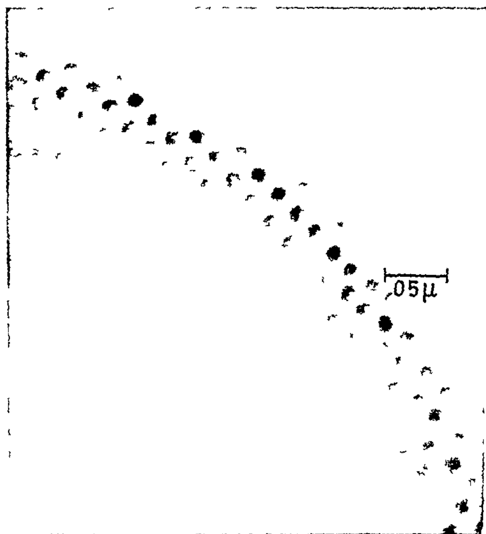
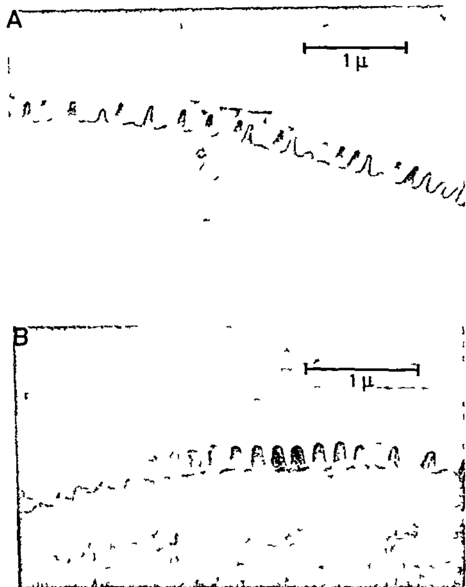


Fig 7 Electron micrograph of the cornea of a mosquito (*Culex*) sectioned oblique to facet surface



F g 8 Electron micrograph of a night moth cornea *(A Prodenia eridiana)* and a butterfly cornea *(B Vanessa urticae)* sectioned normal to the corneal facet surface

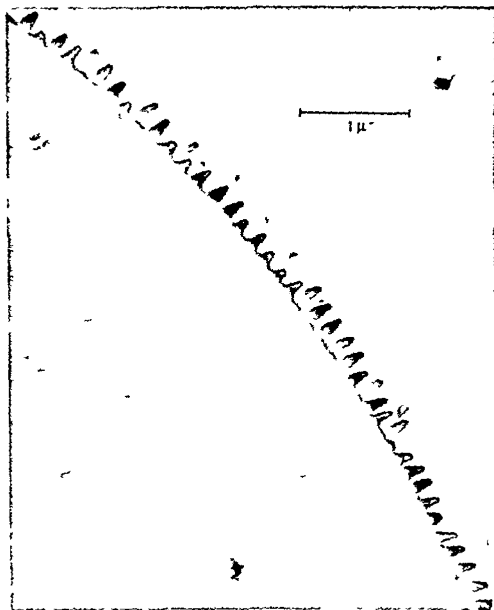


Fig 9 Electron micrograph of a night moth cornea *(P r lema endiana)* sectioned normal to corneal facet surface

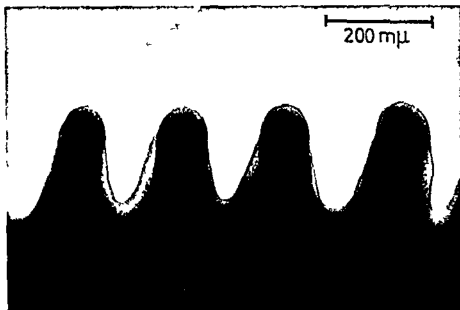


Fig 10 Electron micrograph of four corneal nipples in a night moth *(Laothoe populi)* sectioned normal to corneal facet surface

Fig 11 Electron micrograph of corneal nipple array in a night moth *(Prodenia eridiana)* sectioned tangent to corneal facet and slightly above the surface

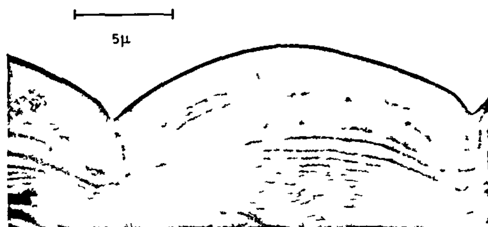


Fig 12 Electron micrograph of a house fly cornea (*Musca*) sectioned normal to corneal facet surface

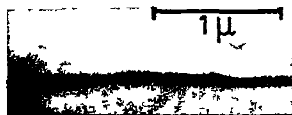


Fig 13 Electron micrograph of a house fly cornea (*Musca*)

PART II

INVESTIGATION OF THE FUNCTION OF THE CORNEAL NIPPLES

1 INTRODUCTION

The electron microscope reveals that the front surface of certain insect corneas is completely covered with minute nipples smaller than a wavelength of visible light and arranged in hexagonal array. On their discovery the function of these nipples was completely unknown to us. It seemed unlikely that this surface structure would be of no significance for the animal taking into consideration the striking regularity of the pattern, its distribution among the arthropods and its dimensions. On the cornea transmitting electromagnetic radiation as it does, it seems curious that there exists an array of structures with dimensions of 1/2 the wavelength of light from the middle of the visible spectrum, since it is a well known dimension for antenna function. On the other hand, one wonders if these irregularities on the front surface of the cornea might impair its transparency and render it like milkglass.

Two kinds of experimental investigations were undertaken to analyse the function of the nipples. First, the properties of scaled dielectric models of the cornea both with and without nipples were systematically investigated using microwaves. Second, properties of various insect corneas were studied by illumination with light. These investigations of the function of the corneal nipples have been briefly described in preliminary notes (Bernhard, Miller and Moller 1963, Miller, Bernhard and Moller 1964).

B MICROWAVE EXPERIMENTS ON MODELS OF THE CORNEA

1 INTRODUCTION

Both light waves and radio waves are electromagnetic radiations, and as such are qualitatively similar When electromagnetic radiation travels through a boundary between two 'transparent' materials in which the propagation velocities are different, some of the energy is reflected and some is transmitted For optics the ratio of the velocity of propagation of light of a given wavelength in free space to its velocity in another medium is the refractive index (n) of that medium For radio waves traveling from free space to a dielectric material the ratio of velocities is equal to the square root of the dielectric constant of the material (ε) The dielectric constant (ε) thus corresponds to the square of the refractive index ($\varepsilon = n$)

The intensity of electromagnetic radiation reflected from the boundary between air and a "transparent material in which the velocity of propagation is different from air is a function of the refractive index of that material in the case of light and of its dielectric constant in the case of radio waves Thus, for light waves in air at normal incidence to the surface of a material with an index of refraction n the intensity of reflected energy r, is expressed by the following equation

$$r = \frac{(n-1)}{(n+1)},$$

For radio waves incident on a dielectric material with a dielectric constant ε the corresponding relationship is

$$r = \frac{(\sqrt{\varepsilon}-1)^2}{(\sqrt{\varepsilon}+1)^2}$$

For both light and radio waves the intensity of transmitted radiation is of course the difference between the incident and reflected intensities

The radiation is expressed here in terms of power, which is usual in the case of light

Not only does the refractive index have its counterpart in the dielectric constant, but also the degree of transparency to light waves has its counterpart in the dielectric losses for radio waves

Because of the equivalences between these different parts of the electro

magnetic spectrum it is only necessary in designing an experiment in which scaled models are used to choose a region of the spectrum for which the model will be of a practical size We chose as a convenient wavelength the region in the neighborhood of 3 cm (corresponding to a frequency of 10 kilomegacycles per second KMC) Since the nipple amplitude and center to-center spacing which is called d (approximately 200 mμ in the insect's cornea) corresponds to an optical path length (n d) equivalent to half the wavelength of light in the middle of the visible spectrum ideally one should build one type of model with 1 5 cm nipples and study its transmittance and reflectance for microwaves of wavelengths from say 0 7 to 7 0 cm For practical reasons we used three microwave generators, each operating in limited wavelength ranges and built a number of models with different sized nipples to complement the generators For this reason the reader will note that the properties of the models are graphed as a function not of wavelength but of a ratio microwave length to nipple dimension (λ/d) This also is an advantage in transposing the scale to the wavelengths of light

II METHODS

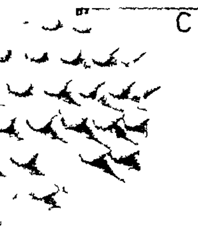
Design and Manufacture of Models

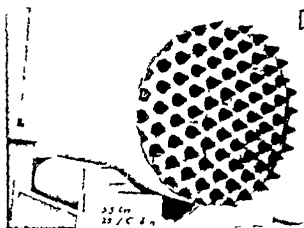
Scaled models of the cornea of the night moth *Prodenia* were constructed on the basis of the electron micrographs In construction of the models conical approximations to the nipples were made The center to-center spacing and amplitude of the cones were chosen to be equal and is referred to as d The base diameter was chosen to be 0 6 d and the radius of curvature of the model s surface to be about 100 d The particular choice of a one to-one relationship between the amplitude of the cones and the center to-center distance implies a simplification and was influenced by the fact that perfect models of certain sizes with no interspace between the cones were found to be difficult to cast The significance of this relationship for the performance of the model is thoroughly treated in the discussion

In the six sizes of models constructed d had the following dimensions 1 0 cm 1 5 cm 2 0 cm 3 0 cm 3 5 cm and, 6 0 cm Fig 14 A and C and Fig 27 show models with three different size nipples Using these models in conjunction with three microwave generators (4 to 6 8 to 12 and 25 KMC see microwave equipment) we measured transmission through the model and reflection from its front surface for ratios of microwave length (λ) to nipple pattern constant (d) of 0 2 to 5 0 These scaled dimensions correspond to light wavelengths of 40 mμ to 1000 mμ for the actual cornea









ig 14 A and I Experimental setup for measuring the transmission of microwaves through scaled dielectric models of the ins ct cornea A shows the transmitting antenna in front of the rippled surface (3 5 cm ripples) of the model (cf Fig 20) and the receiving antenna looking through an opening in a plate of absorbing material which is mounted upon a wheeled platform riding on rails along the geometrical axis behind the model B rear view of the same setup with the model covered by absorbing material C model with 6 cm ripples D model with 3 5 cm ripples containing 25 carbon

The models were scaled both to the correct geometrical dimensions and to the appropriate refractive power and transparency The refractive index of the night moth cornea *(Cerapteryx graminis)* is 1 57 (Carlstrom, personal communication) and qualitatively the cornea seems very transparent to visible light Al o the model had to be made of a material

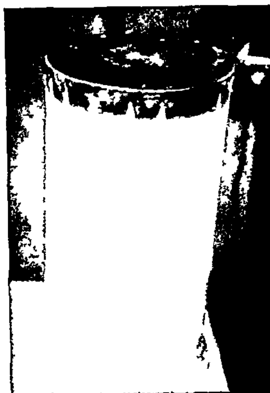
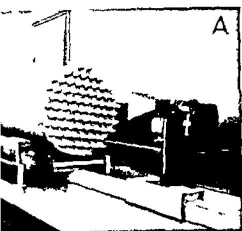
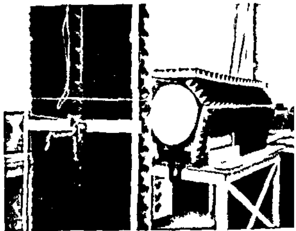


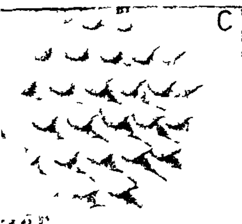
Fig 15 Dielectric model being manufactured The cylindrical wall of the mold has been removed after casting the mold giving the nippled surface is still on the top of the model

that was easily cast and cut After trying a number of materials we chose one that showed a compromise of all these properties a mixture consisting of 75% paraffin and 25% beeswax Its dielectric constant is assumed from published measurements to be about 2 25 which corresponds to a refractive index of 1 5 ($n = \sqrt{\varepsilon}$) This dielectric material was found to be very transparent to microwaves

Plastic negative molds were made from wooden models of the nipple pattern The dielectric models were manufactured by filling these molds with the melted compound of paraffin and beeswax The models were cylindrical with the base representing the front surface of the cornea covered with nipples Since casting a complete model is very time consuming a small thickness of the corneal model surface including the nipples was cast separately and then welded to the cylinder (Fig 15) which had a diameter of about 30 cm and was about 60 cm in length This procedure was found to be convenient since measurements had to be made on hundreds of dielectric models before and after resection of the nipples







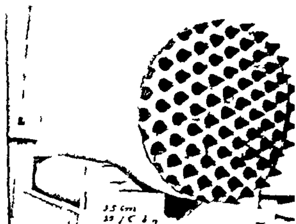


Fig. 14. A and B. Experimental setup for measuring the transmission of microwaves through scaled dielectric models of the insect cornea. A shows the transmitting antenna in front of the nippled surface (3.5 cm nipples) of the model (cf. Fig. 20) and the receiving antenna "looking" through an opening in a plate of absorbing material which is mounted upon a wheeled platform riding on rails along the geometrical axis behind the model. B: rear view of the same setup with the model covered by absorbing material. C: model with 6 cm nipples. D: model with 3.5 cm nipples containing 25% carbon.

The models were scaled both to the correct geometrical dimensions and to the appropriate refractive power and transparency. The refractive index of the night moth cornea *(Cerapteryx graminis)* is 1.57 (Carlstrom, personal communication) and qualitatively the cornea seems very transparent to visible light. Also the model had to be made of a material

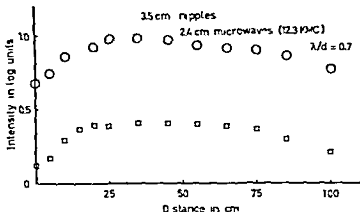


Fig 17 Intensity of transmitted energy in log units plotted as a function of distance in cm from the back of the model along its geometrical axis before (open squares) and after (open circles) resection of the nipples

the model was adjustable but was usually fixed at 90 cm The receiving antenna was mounted upon a wheeled platform riding on rails so that its distance from the back of the model could be easily varied Measurements were taken along the geometrical axis of the model as well as at different angles relative to the axis In a typical experiment the intensity of a given frequency of radiation transmitted by the intact model was measured at 15 places along the geometrical axis Immediately after this the nipples were resected with a sharp machete and the measurements were repeated It was important that the energy of the unavoidable reflections from the walls of the room be small compared to the energy transmitted through the model The energy of this stray field would reach the receiving antenna in a phase relation with respect to the energy transmitted through the model that would be dependent on the distance between the antennae Therefore, a fluctuating reading of the intensity would result as the receiving antenna was moved However the stray energy that reached the receiving antenna was brought to a minimum by arranging absorbent material around the model and behind the receiving and transmitting antennae (See Fig 14 B)

The difference in the two readings obtained before and after cutting the nipples was used as a measure of the effect of the nipple array Since it is a relative measurement, the accuracy is in the order of magnitude of the reproducibility which was found to be about 0 02 log units

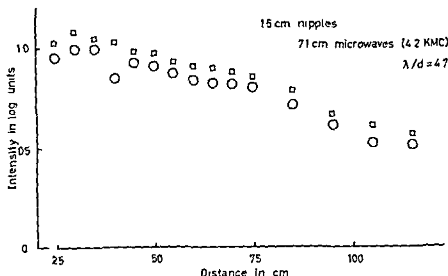


Fig 18 Intensity of transmitted energy in log units plotted as a function of distance in cm from the back of the model along its geometrical axis before (open squares) and after (open circles) resection of the nipples

Results

1 Transmission as a Function of Relative Wavelength

In these experiments the position of the transmitting antenna was fixed at 90 cm on the axis in front of the model and the receiving antenna was moved along the axis behind the model In general the measurements of intensity of radiation along the axis when starting near the back of the model and moving away from the back first rises, then comes to a maximum (focus of the planoconvex lens) and finally shows a gradual decrease

The constancy and reproducibility of the measurements are very good along the axis except for the first few centimeters directly behind the back surface of the model Consequently we usually excluded the first few centimeters and averaged the balance of the measurements Specific experiments are illustrated in Figs 16, 17 and 18 Fig 16 shows the results of an experiment on the transmission of 3 5 cm microwaves through a model with 3 0 cm nipples The graph shows the logarithm of the intensity of transmitted radiation as a function of distance from the back of the model The squares represent measurements taken before resection and the circles after resection of the nipples At this wavelength which is 1 2 times the nipple dimension ($\lambda/d = 1\ 2$) the nipples appear to have no effect on the intensity of radiation transmitted through the model Fig 17 shows the

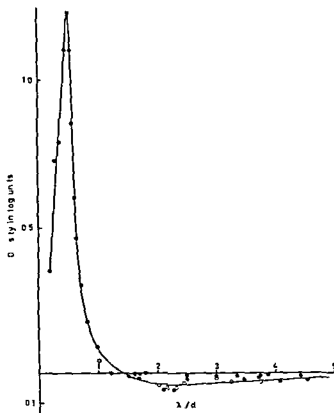


Fig 19 Influence of the nipples on the transmitted energy through the dielectric model as measured on its geometrical axis Density (in log units) caused by the nipples is plotted as a function of wavelength (expressed in nipple dim ns on λ/d) Closed circles represent average values obtained in experiments of the type illustrated in Figs 16—18 Open circles are values obta ned in experiments with the receiving horn fixed at the back of the model

results of a similar experiment but in this case the wavelength is only 0 7 times the nipple constant ($\lambda/d = 0\ 7$) As in Fig 16 the squares represent measurements taken before resection and the circles after resection After the nipples are removed the intensity of transmitted radiation is higher by about half a log unit As seen in the graph this increase in transmitted energy is noted wherever measurements were made along the axis

Finally Fig 18 illustrates the results obtained from an experiment in which the microwavelength is 4 7 times the nipple constant ($\lambda/d = 4\ 7$) In this case after the nipples are resected there is a decrease in transmitted energy for all the measured points along the axis These results are typical for experiments in which λ/d is greater than 1 5

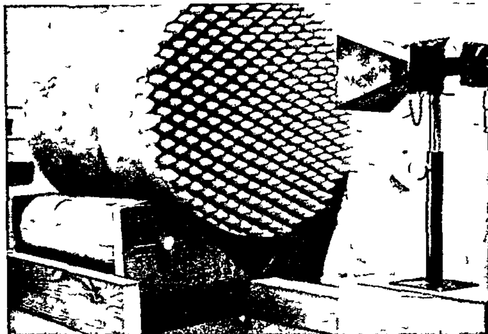


Fig 20 Transmitting antenna in front of a model with 3 cm nipples close together on the front surface

We performed this type of experiment for values of λ/d of 0 2 to 5 0 The results of these experiments are summarized in Fig 19 The average value of the intensity in log units of transmitted radiation through each model with nipples is subtracted from the average value for the model without nipples These calculated differences are graphed as a function of λ/d Thus Fig 19 gives an indication of the optical density' of the nipples in log units for points along the geometrical axis The curve shows that the nipples have a peak density of more than 1 2 log units at λ/d = 0 5 With increasing values of λ/d the density decreases and for values of λ/d over 1 5 the nipples cause a decrease in density, i e an increase of transmitted energy in other words, the presence of the nipples on the model *results in a small increase in transparency for values of λ/d greater* than 1 5

It is evident that for the conditions described in the foregoing experiments the nipples increase the "optical density of the model for relatively short wavelengths and tend to increase the "transparency of the model towards the relatively longer wavelengths

It is interesting that for the relatively longer wavelengths the nipple array makes the model more transparent even though with the nipples attached the radiation has to pass through more material This deserved

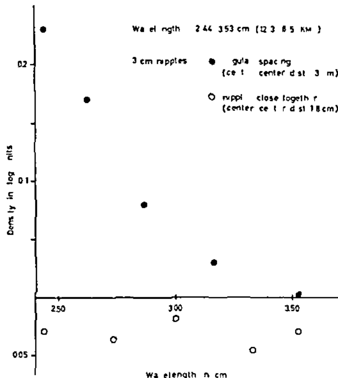


Fig 21 Influence of the nipples on the transmitted energy through the dielectric model as measured on its geometrical axis Closed circles 3 cm nipples and regular spacing Open circles 3 cm nipples close together Density (in log units) caused by the n pples is plotted as a *function of wavelength in cm*

closer study Therefore in some experiments we fixed the receiving horn to the back of the model to increase mechanical stability and reduce the *uncertainty of measurement and performed the experiment on the roof* of the laboratory in order to minimize the effects of scattered radiation The results are given by the open circles in Fig 19

2 Transmission as a Function of Physical Properties of the Model

We designed two types of experiments in order to elucidate the mechanism of action for the increased density of the model with nipples in the relatively shorter wavelengths *First* we changed the relative dimensions of the amplitude versus center to-center spacing of the array and in another series of experiments altered the dielectric constant of the material from which the nipples were cast *Second* we measured the influence of the nipples on the optical density as a function of angle of incidence of the radiation

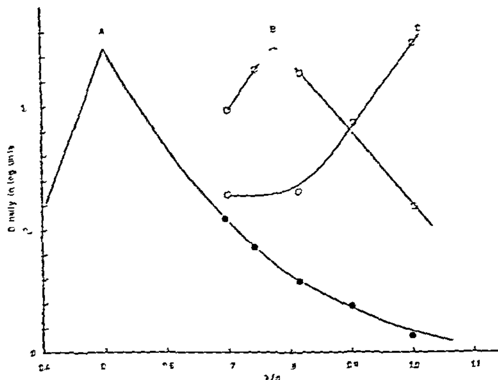


Fig 22 Curve A shows the influence of the ripples on the transmitted energy through the dielectric model as in Fig. 19. Density in log units caused by the ripples is plotted as a function of wavelength (expressed in ripple dimension λ/d). Closed circles, the same with the model in reversed position (back of the model facing the transmitting antenna). Curves B and C show the result obtained on models with ripples containing 2, % (B) and 50 % (C) lampblack.

Relative Dimensions of the Array In order to qualitatively determine the relative importance of the various dimensions of the array we both shortened and lengthened the center-to-center distance keeping the amplitude the same (Fig 20). Fig 21 shows the results obtained using a model on which 3 cm ripples were placed as close together as possible (center-to-center distance 1 8 cm). For the range of 8 5 to 12 3 KMC we found that when the center-to-center distance of the 3 cm tall ripples was 3 cm, the aforementioned increase in density was observed (filled circles) while for the altered model we observed a decrease in density (open circles) It would therefore appear that in moving the ripples closer together, the curve shown on Fig 19 is shifted to the left.

Dielectric Constant It might be expected that changing the physical properties of the dielectric material would affect its properties to microwaves To explore this problem we mixed lampblack (powdered carbon) in the compound used to cast the ripples We experimented on models with



Fig 23 Experimental arrangements on the roof of the laboratory (see text) for studying the transmission through the dielectric model at different angles of incidence Transmitting antenna in front of the metal diaphragm through which the rippled surface of the model is looking

ripples containing 1% 10% 25% and 50% lampblack by weight A model carrying ripples with 25% carbon is shown in Fig 14 D The addition of the lampblack increased the dielectric constant to about 4 for 25% lampblack and 5 for 50% lampblack (Haapanen and Kuhmonen, personal communication) It should be noted that the addition of carbon probably also altered the loss factor or transparency although we were not able to measure this At any rate, as is shown by curves B and C in Fig 22, the addition of carbon shifts the peak density towards the longer wavelengths Curve A in Fig 22 is essentially the same as the left hand portion of Fig 19

Angle of Incidence In Fig 22 the filled circles represent values obtained in experiments in which the rippled surface of the model faced the receiving antenna rather than the transmitting antenna As seen in Fig 22 the values taken in these experiments fall on the curve This confirms the well known principle in optics of the reversibility of ray tracing

Because of the principle of reversibility it makes no difference for these transmission measurements whether the inclination of the receiving or transmitting antenna is altered Both types of experiments were performed and we have chosen the results indicated in Fig 24 to illustrate our findings

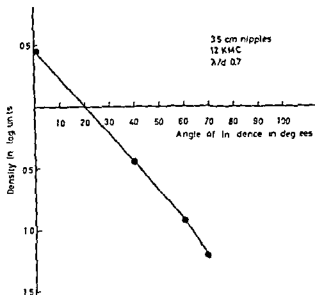


Fig 24 Influence of the nipples on the transmitted energy through the dielectric model Density (in log units) caused by the nipples is plotted as a function of angle of the transmitting horn (zero represents normal incidence) The receiving horn was fixed at the back of the model

obtained by changing the angle of the transmitting horn with respect to the geometrical axis of the model In these experiments the receiving horn was not moved on the geometrical axis but rather was fixed to the back of the model at its center Because of scattered radiation and diffraction, which is caused by the small aperture of the lens, special precautions had to be taken The reliability of the measurements was increased by taking them in a relatively echo free situation on the roof and by constructing a metal diaphragm at the front surface of the model (Fig 23)

On the abscissa of Fig 24 is given the angle of the transmitting horn in degrees with zero representing normal incidence On the ordinate is the optical density of the nipples These measurements were made at $\lambda/d =$ 0 7 where for zero degree s incidence there is an increase in density of about 0 5 log units (also cf Fig 19) As the angle of incidence increases the density of the ripple array decreases Further, for values of λ/d approaching 1 0 the decrease in density of the nipples becomes increasingly smaller for a given angle of incidence (not depicted)

Thus it is found that the observed increase in density along the geometrical axis does not correspond to an absolute net decrease in transmitted energy Rather the energy is redistributed into zones of greater and lesser intensities and we conclude that the measured increase in density on

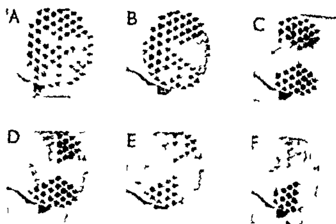


Fig 25 Polarization experiment A the surface of the model with 3 5 cm ripples B—F show the different sectors which were denuded in the order given by the numbers in the inset of Fig 26

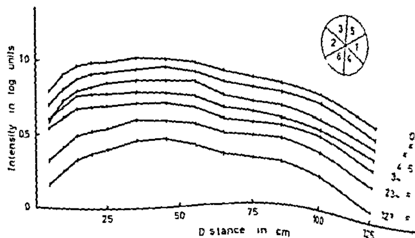


Fig 26 Intensity of transmitted energy in log units plotted as a function of distance (in cm) from the back of the model (along the geometrical axis) before (curve 0) and after successively denuding the sectors marked 1—6 (inset) The numbers show the order in which the sectors were denuded and the numbers to the right of each curve indicate the sectors which in each case remained intact before the measurements were made

the geometrical axis at the relatively shorter wavelengths is a result of an interference phenomenon caused by the hexagonal array of ripples

Polarization We tried a number of experimental approaches to find out whether transmission of radiation through the hexagonal array of ripples is a function of the plane of polarization of the microwaves. For technical reasons we found it difficult to change the plane of polarization by rotating either the model or the antennae We achieved the most

ducible results by measuring transmission through the model as a function of the removal of nipples in patterns of sectors with different angles (Fig 25)

The results of such experiments are illustrated by the data presented in Fig 26 from an experiment in which the transmission of 2 4 cm micro waves through a model carrying 3 5 cm nipples containing 50% carbon was studied ($\lambda/d = 0\ 7$) The lowest curve in Fig 26 shows the intensity of transmitted radiation as a function of distance from the back of the model before resection of any of the nipples Similar measurements were then repeated after consecutive stepwise resection of the nipples within sectors 1 through 6 as depicted on the inset of Fig 26

Table 1

Sector	Number of nipples per sector	Density in log units per sector	Density in log units per nipple
1	17 5	0 155	0 009
2	16 5	0 138	0 008
3	8 7	0 072	0 008
4	8 7	0 068	0 008
5	12 5	0 085	0 007
6	12 5	0 114	0 009

The sectors were denuded in the order indicated by the numbering (cf Fig 25) Thus the numbers next to each curve in Fig 26 indicate the sectors which in each case remained intact before the measurements were taken The uppermost curve (marked 0) was obtained after resection of the nipples in sector 6, that is, after removal of all the nipples (not depicted in Fig 25) As shown by the curves there is a stepwise increase in transmitted energy all along the geometrical axis each time a sector is denuded The number of nipples in each sector is given in Table 1, as is the optical density of each sector and the average optical density per nipple for the indicated sectors

For this experiment the average optical density per nipple is very close to 0 008 log units with a variation of plus or minus 0 001 log units Because of uncontrollable asymmetries in the model and in the alignment this amount of variation of the data cannot be interpreted as being significant These results, therefore do not allow one to conclude that the measured density of the nipples is a function of the plane of polarization of the radiation for the relatively shorter wavelengths

While the experiment described here was performed on a model with

nipples containing lampblack, the results were found to be equally applicable to models with nipples not containing carbon For such models (without carbon) the results of these polarization experiments apply to the region where λ/d is less than 1 5 (see Fig 19)

We have no information on the effect of the plane of polarization on models for λ/d over 1 5 It will be recalled that where λ/d is greater than 1 5 there is an increase in transparency Since this increase in "transparency" is so small, it would be impossible under the present experimental conditions to determine whether it is a function of the plane of polarization

IV REFLECTION MEASUREMENTS

Procedure

Accurate measurements of the energy reflected from the front surface of the model are technically more difficult to obtain than are the transmission measurements The reason for this is that the quantity of radiation reflected from a dielectric material (r) is a function of its dielectric constant

$$r = \frac{(\sqrt{\varepsilon} - 1)^2}{(\sqrt{\varepsilon} + 1)}$$

Thus for our models *without* nipples the reflection is 4% since

$$r = \frac{(\sqrt{2\ 25} - 1)^2}{(\sqrt{2\ 25} + 1)} = 0\ 04\ (i\ e\ \ 4\%)$$

Thus the intensity of the stray field is proportionately much higher for this type of measurement In order to overcome this difficulty we constructed a platform on the roof of the Physiology Building of Karolinska Institutet and located the equipment in a relatively echo free situation The experimental setup is shown in Fig 27 The horns are placed side by side facing the front surface of the model and close to its geometrical axis The energy that is picked up by the receiving horn must be only that reflected by the model and direct transmission from one horn to the other has to be kept to a minimum In this case the direct transmission was of the order of the noise level which was about two log units and always more than one log unit below the reflected energy measured The noise level was estimated by removing the model and reading the output of the detector

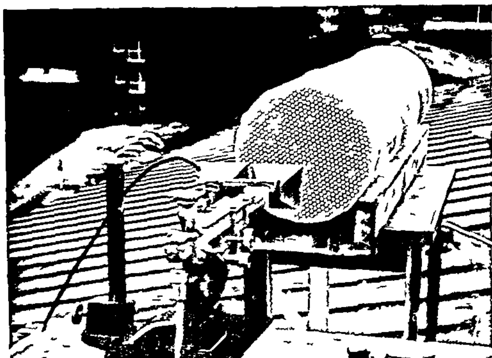


Fig 27 Experimental setup at the roof of the laboratory (echo free situation) for measuring the reflection from the surface of a model with 1 5 cm nipples Transmitting and receiving antennas placed side by side in front of the long dielectric model

The microwave generator was placed in the attic and the energy was fed to the transmitting antenna via a 3 cm waveguide The distance between the antennae and the front of the model was about 70 cm As seen in Fig 27 the model was three times longer than that used in the transmission measurements and in addition the back was cut obliquely The extension of the model's length provided greater absorption of the wave that was reflected from the backside while the oblique back tended to reflect out of the side of the model rather than directly into the receiving antenna *In order to investigate the effect of the nipples on reflection, measurements* were made before and after resection of the nipples in the same way as in the transmission experiments The accuracy of the measurements was the same as in the transmission experiments

It should be noted that the character of radio waves usually differs in one respect from that of light waves Radio waves consist of single frequencies (sine waves) while the energy in light waves commonly is spread over a broad frequency band (continuous spectrum) In experiments with models where light is substituted by radio waves this difference between light and radio waves may influence the resulting measurements In such

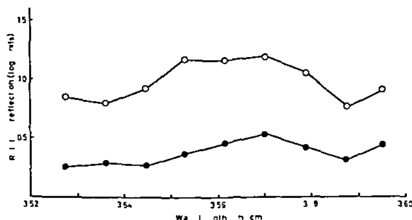


Fig 28 Relative reflection (in log units) plotted as a function of wavelength before (closed circles) and after (open circles) resection of the nipples (1 5 cm)

a case the radio wave measurement is not a good representation of the situation for light unless the results of measurements at a number of wavelengths are averaged to represent a continuous spectrum To be a good representation of the optical situation it is necessary to measure the transmission or reflection of the model for a number of wavelengths within a limited range The average values for this wavelength range of microwaves are then comparable to measurements on optical systems with light

Results

As noted above we used relatively long models in these experiments since a long model attenuates the wave reflected from the backside more than does a short model The reflection measured at the front is the resultant of the wave reflected from the front side and that reflected from the backside If these two waves are in phase they add if they are of opposite phase they subtract The phase angle of the wave reflected from the back side in relation to that reflected from the front side is dependent on the distance in wavelengths from the front side to the backside which varies with the frequency of microwaves These variations are therefore periodic and to approximate the situation with light it is only necessary to average the measurements over a single period

The results from an experiment using a model 180 cm in length with 1 5 cm nipples and with an obliquely cut back are graphed in Fig 28 On the abscissa is the wavelength in cm and on the ordinate is the logarithm

of the reflected intensity The closed circles show the reflected intensity from the model before resection of the nipples for various wavelengths within a limited range (3 54—3 61 cm), and the open circles represent values obtained after resection of the nipples At each wavelength there is a difference between the two readings, showing that the nipple array reduces the reflection from the front surface of the model

It is noted that although the difference between the open and filled circles is relatively constant, both curves show a periodic variation In order to exclude the possibility that this variation in the measured reflection is due to variations in the radiated energy from the transmitting horn or to changes in the sensitivity of the receiver, a plane aluminum sheet giving total reflection was placed on the model s front surface and the same measurements were then made There was no periodic variation in the readings from the metal plate, indicating that the character of the setup is uniform over this range and that the aforementioned periodicity is there fore not due to imperfections in the measuring equipment As is explained above, a periodicity would result from interference between the reflected waves from the front and back surface of the model This implies that in this experiment in spite of the precautions taken, there is still some re flection from the model s back surface The periodicity is correlated with the relation between the length of the model and the wavelength If the backside reflection was not attenuated the measured reflection at the front surface of the model without nipples would approach zero at wavelengths where the wave reflected from the backside appears at the front side equal in amplitude and opposite in phase to the wave reflected from the front side In the experiment illustrated in Fig 28 such dips in the curve would occur around wavelengths of 3 53 and 3 59 cm At wavelengths where the backside and frontside reflections appear in phase they add and the measured reflection would approach 8% of the incident energy (at 3 56 cm in Fig 28) The result would thus be fluctuations in the curve covering several log units If on the other hand there would be no backside reflection the curve would be flat The fact that the size of the variations in the mea sured reflection as a function of wavelength is less than 0 5 log units indi cates that the backside reflection into the model is considerably attenuated Therefore the average difference between the measured reflection before and after cutting the nipples gives a satisfactory measure of the decrease in reflection caused by the nipples

Fig 29 shows the results of experiments on models with and without the nipple array as a function of λ/d Each of the points on this graph represents the difference in the mean of measurements made over one period The calculation of the mean values was performed on a linear

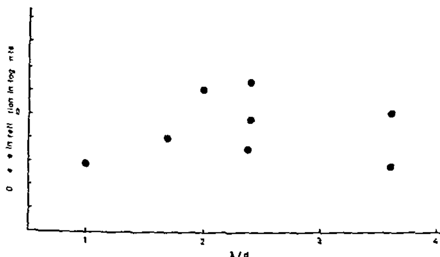


Fig 29 Decrease in reflection (in log units) caused by the nipples plotted as a function of wa elength (expressed in nipple d mension λ/d)

(energy) scale The values of the average energy in Fig 29 are given in log units It is seen that the nipple array causes a decrease in the reflected intensity of about 0 5 log units for the range covered We do not have enough values to make a conclusion about the exact form of the curve showing the decrease in reflection in relation to λ/d which can however be derived from other considerations (see Section D)

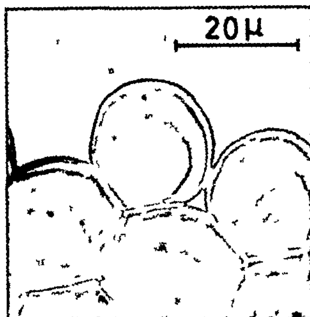


Fig 30 Slice of a night moth cornea *(Endromis)* showing a group of facets Black dot illustrates location of a 1 μ spot to be illuminated with spectral light in the microspectrophotometric experiment

C SPECTROPHOTOMETRY

The experiments with microwaves on models of Lepidopteran corneal facets have shown that (1) for radiation wavelengths less than the height or center to center distance of the nipples there occurs an interference phenomenon that results in zones of greater and lesser intensities of transmitted energy, (2) for wavelengths longer than the nipple dimensions the nipples act to decrease reflection from the front surface of the model and to increase the transmission through the model On the basis of this information, we now turn to experiments in which some of the properties of actual corneas are measured as a function of wavelength of light

1 TRANSMISSION OF LIGHT

Methods

In these experiments we used the corneas from two kinds of moths *(Smerinthus ocellata Endromis versicolora)* For most of the experiments a full thickness slice of cornea usually under a square millimeter in area was excised using a razor blade The specimen was mounted on a quartz

slide and a quartz coverslip was gently sealed over it using melted paraffin wax The preparation was placed in a Zeiss universal microspectrophotometer in which the optical density of the specimen was measured using spectral illumination of small bandwidth for the range of 240 to 700 mμ The illumination was usually confined to a 1 or 10 micron diameter spot, usually located on the facet by means of the same glycerine immersion objective that the measurements were made with (Fig 30) The usual routine followed was first to measure the relative amount of transmitted light through the cornea for the spectrum and then measure the relative transmission of light in the absence of the cornea From these data we calculated the optical density (extinction) of the cornea which is expressed as the logarithm of the ratio of incident (absence of cornea) to transmitted light

Results

1 Transmission as a Function of Wavelength

We photographed corneal slices using transmitted monochromatic light in an effort to observe the corneal nipples Photographs taken with wavelengths as short as 250 mμ failed to give a definite indication of the nipple pattern

After this unsuccessful attempt to visualize the nipples we measured the density of the specimen transilluminated by a spot that was 1 μ in diameter The results are shown in Fig 31 where the optical density in log units is graphed as a function of wavelength from 250 mμ to 700 mμ The transparency of the cornea is relatively constant for most of the range between 320 mμ and 700 mμ whereas under 320 mμ it decreases sharply this effect peaking at 280 mμ

There is an obvious superficial resemblance between Fig 31 and Fig 19 (experiments with models) and one might ask if the observed increase in optical density to light corresponds to the increase in density to microwaves that is a property of the nipple array as measured on the geometrical axis In examining this question one notes discrepancies between the results of these two experiments First the increase in density caused by the nipples in the microwave experiments has its peak effect where $\lambda/d = 0.5$ If the scaling of the model is correct this corresponds to a wavelength of light of 100 mμ If the nipple pattern is in fact responsible for the increase in density to light as has been shown to be the case for microwaves it would mean that the scale factor is wrong by almost three times And second it should be noted that the experimental procedure in the microwave experiments (Fig 19) in which the average intensity was measured

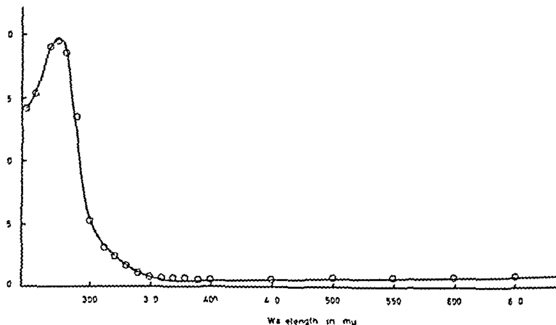


Fig 31 Results of a spectrophotometric experiment showing the density in log units of a night moth cornea (*Endromis*) as a function of wavelength (illuminated spot 1 μ in diameter)

along the model's axis may not correspond exactly with the experimental conditions in the spectrophotometric experiments. For these reasons it seems probable that the resemblance between Fig 31 and Fig 19 is fortuitous but in order to learn with certainty whether or not the nipple array is responsible for the increased density in the near ultraviolet we measured the density contributed by different layers of the cornea.

We measured the optical density of full thickness moths' corneas, of corneas from which a thin slice containing the nipple array had been removed, and also of the thin slices *carrying the nipple array*. A partial thickness layer (B in Fig 32 and inset in Fig 34) of the front surface of a moth cornea was removed with a razor. The underlying part of the cornea (C, in Fig 32 and inset in Fig 33) was likewise removed from the eye. Density measurements were carried out on both slices with a 1 μ diameter spot at the places indicated by the symbols on the drawings. The results of these measurements are graphed in Figs 33 and 34, where results for the bottom part of the slice are given in 33 and the top part of the slice in 34. The measurements marked by open and filled circles and by

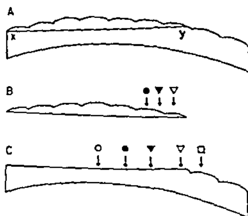


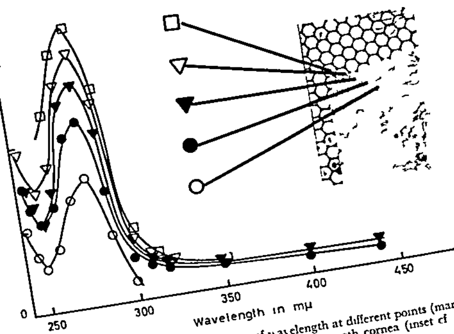
Fig 32 A schematic drawing of a piece of a night moth cornea from which the top part (B) containing nippled facets was removed from the underlying part (C) by cutting (x—y) with a sharp razor blade The circles, squares and triangles indicate the various spots at which the relative corneal density for spectral light of different wavelengths was measured in experiments illustrated in Figs 33 (bottom part) and 34 (top part)

filled triangles in Fig 33 were made on the part of the underlying slice (see inset) where the corneal thickness was progressively increasing and the nippled surface was absent The open triangles mark results obtained on the border region where the razor separated the nippled surface from the underlying corneal thickness while the open squares mark measurements made on the full thickness of the cornea (cf Fig 32 C) The graph in Fig 33 shows that the peak of optical density at 280 mμ is independent of the presence of nipples and that the magnitude of this peak increases with increasing thickness of the corneal slice In Fig 34 the closed circles closed and open triangles represent measurements made on various parts of the cornea containing nipples and of progressively increasing thickness (see Fig 32 B) The curves show that even though the nipple array is present for the open circle measurements there is very little increased density and for the increasingly thicker parts that also have nipples the density seems to increase along with the corneal thickness

Thus we conclude that the increased density of these corneas that has been observed to occur at wavelengths in the near ultraviolet is not in any way related to the corneal nipple array but rather is a result of some other properties of the corneal chitin

2 Quality of the Image

Moth corneas carrying the nipple array transmit radiation from the visible spectrum extending to the near ultraviolet We know this from spectrophotometric measurements described in detail in the previous section We also know from the results of the experiments with scaled models



g 33 Optical density in log units as a function of wavelength at different points (marked ith triangles circles and squares) at the bottom part of the moth cornea (inset cf Fig 32 C)

Fig 34 Optical density in log un ts as a funct on of wa elength at different points (marked with circle and triangles at the top part of the moth cornea (inset cf Fig 32 B)

that the nipple array decreases reflection from the model s front surface and slightly increases the model s transparency Still, one may wonder whether or not the unevenness of the front surface of the cornea impairs the quality of the transmitted image

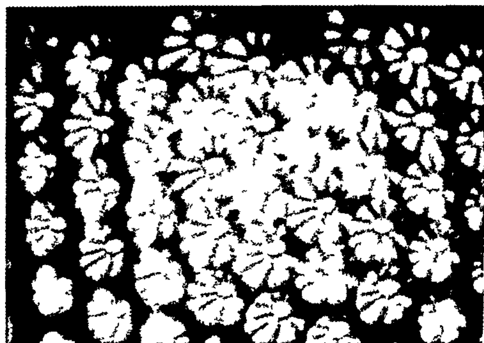
That the corneas of many compound eyes transmit images has been long known In this regard Leeuwenhoek s experiments are of considerable interest Speaking of the dragon fly compound eye he said "I cleared away the many vessels which fill the inside of the tunica cornea, or horney coat of the eye, leaving only the tunica cornea remaining When I removed this tunica cornea a little from the focus of the microscope, and placed a lighted candle at a small distance so that the light of it must pass through the tunica cornea, I then saw through it the flame of the candle inverted, and not a single one but some hundreds of flames appeared to me, and these so distinctly (though wonderfully minute) that I could discern the motion or trembling in each of them

Each of the corneal facets, then, forms a reduced and inverted image located within the eye and close to the cornea

Does this same situation apply to corneas which carry the nipple array? Yes, according to the authority of Leeuwenhoek "I have frequently examined the flying insect moth or butterfly produced from the aurelia or chrysalis of the silk worm, and, I placed before the microscope one of those organs of sight, which in this animal is commonly deemed one eye When this little part, cleared from the optic nerves within it was placed before the microscope all the surround ing objects were clearly to be seen through each of the small optical organs though wonderfully diminutive for the great tower or steeple of our new church in Delft, which is 300 feet high and about 750 feet distant from my house when viewed through any of these optical organs, appeared no larger than the point of a small needle seen with the naked eye (see The Select Works of Antony van Leeuwenhoek 1800)

We have confirmed Leeuwenhoek s observations by photographing a spoked wheel pattern through the corneal facets of a night moth we know to have the nipple array The inner contents of the eye, including the crystalline cones had been carefully removed Fig 35 A shows the re sulting images and Fig 35 B is a reproduction of the pattern used The corneal array does not appear to impair image formation

The importance of image formation for the compound eye is a subject for conjecture, and not germane to the central problem of this paper But it would seem worthwhile to note here that according to the classical mosaic theory of compound eye vision postulated by Johannes Müller (1826) each ommatidium receives an amount of light dependent on its



A



B

Fig 35 A images formed by the facets of the moth s corneal facets of a picture of a spoked wheel pattern B photomicrograph of the rhabdom of the *Limulus* eye (Miller 1954)

entrance aperture and the eye therefore sees a coarse erect image composed of these spots, not multiple inverted images of great detail as may be supposed from Leeuwenhoek s observation This is somewhat at variance with anatomical knowledge of the sensory portion of the ommatidium which we know to be composed of a number of innervated cells and hence possibly capable of more detailed vision than expected by Muller s

theory In fact recent work of Kuiper (1962) Burtt and Catton (1962) and Rogers (1962) suggests that greater resolution is possible though not necessarily because of direct perception of the diminutive inverted images According to Burtt and Catton and Rogers the visual acuity achieved by insects may result from their ability to perceive diffraction images resulting from the small separation of the tiny corneal facets It may be that under specified conditions the nipple array would contribute a component to these complex diffraction effects But for our present level of understand ing we only wish to draw attention to the fact that the nipple array does not interfere qualitatively with the ability of the corneal facets to form images

II REFLECTION OF LIGHT

If one compares the reflection of light from the eyes of insects that do have corneal nipples with those that do not one may observe a bright mirror like reflection from the latter that is never present in the former This is illustrated in Fig 36 where an excised bee eye side by side with a moth eye are photographed by their reflected light The bright specular reflection from the bee eye which does not have corneal nipples is appa rent, and its form is due to the shape of the cornea Because of differences in the shape of the corneas of different insects and of differences in the underlying pigmented structures photographs such as Fig 36 do not give a reliable quantitative measure of the reflection factor We therefore at tempted to make measurements under better controlled conditions

In the microwave experiments one has the advantage of being able to use the same model for measurements of transmission and reflection be fore and after removing the nipples This is not possible for the spectro-photometric reflection measurements because removing the nipples of the actual cornea also changes the radius of curvature of the facet surfaces Therefore we compared the reflection from the corneas of animals that do and do not contain nipples Further we chose corneas of similar dimen sions and shape flies (*Musca* and *Calliphora*) whose corneal front sur faces are smooth (even though there is an underlying nippled substruc ture, see page 20) and moths (*Sphynx* and *Endromis*) that do have the fully developed nipple array

Methods

Pieces of the corneas of the animals were excised and their back sur faces were freed from adhering tissue These corneal fragments were then floated on india ink front side up and observed and photographed by light



Fig 36 Bee eye (to the left) and night moth eye (to the right) photographed side by side by their reflected light

of varying spectral composition In some experiments they were photographed *in the light of a mercury vapor lamp used in conjunction with* appropriate filters (Jena, Glas, Schott and Gen) to pass selected mercury spectral lines For each wavelength pictures were taken with and without a Bausch and Lomb 0 6 log units neutral density filter which previously had been found to be relatively flat in density for the range studied The densities of these photographs were then compared by means of a photocell and calibrated optical wedges by using a microvoltmeter as a null instrument In these measurements corneal areas including about 15 facets were used

Results

Fig 37 illustrates the appearance of fly and moth corneas on india ink by reflected light from a tungsten bulb In Fig 37 the fly's cornea (middle) reflects more light than the corneas from the two different kinds of moths that flank it Experiments of this kind indicate it is reasonable to assume that this difference in reflection is due to the presence or absence of nipples A test of this assumption was made by carefully wetting the moth corneas with aqueous detergent solution using a fine brush When

Fig. 37. A piece of a fly cornea (middle in A and B) and two pieces of a moth cornea (left and right in A and B) floating on Indian ink photographed by their reflected light from a tungsten bulb some minutes after removal from the eye (A) and some hours later (B).

this was done the moth and fly corneas were indistinguishable, probably because fluid filled the spaces between the nipples In preparations such as depicted in Fig 37 one sometimes notices that on first floating the moth corneas, there is very little difference between the reflected light from these and fly corneas This could be due to wetting by surface tension forces or condensation on the nipple array At any rate, within a few minutes the moth corneas reflect a dim reddish light in comparison with the bright white reflex of the fly cornea (A) After some hours, or occasionally a day or more, the moth corneas frequently have a dim bluish appearance by reflected light (B) The reasons for this are unknown It may be due to a continuing drying process A further intangible in these observations is the unavoidable reflection from the interface between the india ink and the backside of the cornea

Measurements made on preparations similar to Fig 37, which preferentially reflect bluish light, are given in Table 2 The difference in reflection between the moth and fly corneas is given in log units for five mercury vapor spectral lines On the average these readings indicate a difference of about half a log unit Because of the unavoidable reflection from the back surface of the cornea, it seems likely that these readings are somewhat too low Additional observations were made at 700 mμ using a tungsten lamp and second-order interference filters Comparison of fly and moth corneas shows that even at this wavelength the reflection is smaller from the moth cornea

The results of these experiments agree with those of the microwave experiments and lead to the conclusion that the corneal nipple array reduces the reflection of light from the front surface of the cornea

Table 2

Difference in intensity between moth and fly corneas in reflected light of different wavelengths

Wavelength in mμ	Difference in intensity in log units
589	0 60
546	0 56
436	0 47
409	0 68
366	0 37

D COMPUTATIONAL MODELS ON THE MECHANISM OF ACTION

I CALCULATIONS OF THE REFLECTION FROM A SURFACE COVERED WITH THE NIPPLE ARRAY

It is evident both from the microwave experiments on models and from the spectrophotometric measurements on the actual cornea that the nipple array decreases the reflection and increases the transmission over a broad wavelength range

The mechanism of this action of the nipple array was demonstrated by numerical calculations performed on a digital computer (BESK) These calculations were based on the following considerations

It is a well known fact in optics that reflection from a surface can be diminished by a coating which makes the transition between the two materials gradual with regard to refractive index (see e g , Blassie 1950) This principle is also commonly used in microwave technology (see Ragan 1948) and in acoustics to decrease reflection from walls (anechoic chambers) and in many other situations Because the nipples are of transparent chitin very regular of a particular configuration, and are also small with respect to a wavelength of light each individual nipple is invisible to incoming light waves The "coating containing the nipple array thus appears homogenous in that the individual nipples are not "discerned The nipples are roughly conical and therefore this coating is not homogenous for refractive index The proportion of the corneal material to air changes from 100% at the nipple base to 0% at the very tips of the nipples In this way the nipple array may provide a smooth transition between the refractive index of the cornea and that of air

We have therefore calculated the reflection from a surface consisting of such a nipple array The nipples are assumed to be conical, so that the refractive index of the array changes from the bases of the cones to their tips We calculated the reflection from the plane of the tips of the nipples This was done by dividing the coating into 20 layers of equal thickness The refractive index is calculated in the plane in front of each layer beginning at the solid cornea where it is 1 57 The refractive index N_1 in the plane in front of the first layer is

$$N_1 = n_1 \left[\frac{1\ 57 \cos(k_1 l) + j n_1 \sin(k_1 l)}{n_1 \cos(k_1 l) + j\, 1\ 57 \sin(k_1 l)} \right]$$

where l is the thickness of the layer (10 mμ) n_1 the refractive index of the first layer $j = \sqrt{-1}$ and $k_1 = 2\pi/\lambda_1$ where $\lambda_1 = \lambda/n_1$ The refractive index in this plane is calculated for a number of wavelengths in the

range of λ/d between 0 5 and 10 The refractive index in each following plane is similarly calculated for the different wavelengths so that the refractive index in the plane in front of the twentieth and final layer (the layer that borders on air) is

$$N_0 = n_0 \left[\frac{N_{19} \cos (k_0 l) + j\, n_{20} \sin (k_0 l)}{n_0 \cos (k_0 l) + j\, N_{19} \sin (k_0 l)} \right]$$

where $k_0 = 2\pi/\lambda_0$

The energy of the wave reflected from the plane at the tips of the nipples is then

$$r = \left(\frac{N_0 - 1}{N_0 + 1} \right)^2$$

Calculation using 20 layers gives a valid approximation to a smooth transition in this case

When such calculations are applied for microwaves and dielectrics (rather than light and transparent media), the refractive index is commonly expressed in terms of its analog characteristic impedance, which is the square root of the ratio of permeability (1 in this case) to permittivity (dielectric constant ε) and hence simply the inverse of the refractive index The term characteristic impedance is equally applicable to light and radio radiations

Curve A in Fig 38 illustrates the results of such calculations for a case where the relation between the spacing and the amplitude of the nipples is that of the models used in the microwave experiments On the ordinate is the decrease in reflection and on the abscissa λ/d

A comparison between Fig 29 and Fig 38 A shows that there is a fairly good agreement between the results obtained in the experiments on the models and the calculated results obtained by using the nipple dimensions employed in the model experiments, there being a decrease in reflection over the wavelength range investigated with a smooth maximum of about 0 5 log units

We therefore conclude that the *nipple array acts as an impedance transformer* which matches the characteristic impedance (inverse of refractive index) of air to that of the lens material (*i e* chitin) over a broad wavelength range

As mentioned in the anatomy part it is rather difficult to form a true notion of the three dimensional reconstruction of the nipple pattern With a given amplitude (d) of the nipples the center to center distance may be larger or smaller than d In the insect cornea the center to center distance

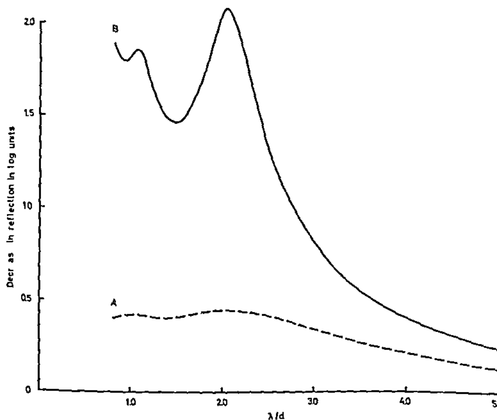


Fig 38 A calculated decrease in reflection as a function of λ/d calculated for a model with a nipple array of the same type as in the microwave experiments (center to-center distance = amplitude = d) B the same for a model with nipples placed close together (center to-center distance = 0 6 times the amplitude = 0 6 d) See text

of the nipples is certainly not larger than the amplitude (see page 11) and it is interesting to note that the theoretical calculations reveal that a center-to-center distance larger than d results in a decrease in the antireflective power A close study of the electronmicroscopic pictures revealed that in certain parts the center to center distances are less than d and that the nipples may even be placed as close to each other as possible (center to center distance = 0 6 d)

In order to find out if such a nipple array is superior in antireflection to that chosen for the model experiments we have calculated the decrease in reflection caused by a pattern with nipples placed close together Curve B in Fig 38 shows that an array with nipples placed close together would be expected to reduce the reflection as much as 2 log units at a wavelength twice the amplitude of the nipples

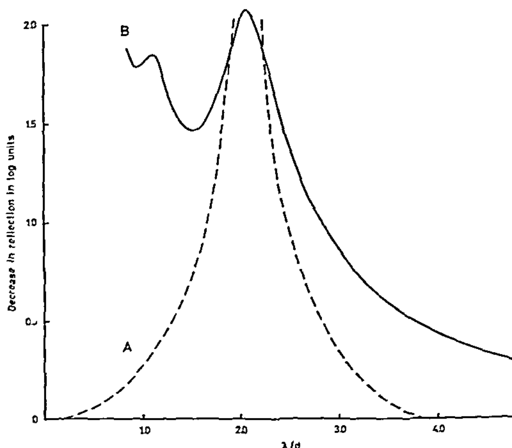


Fig 39 A calculated decrease in reflection as a function of λ/d caused by a quarter wave plate, the thickness of which was chosen to be equal to half the amplitude of the nipples ($d/2$) B the same for a nipple array with nipples placed close together (same as curve B in Fig 38) See text.

II COMPARISON BETWEEN THE ANTIREFLECTION POWER OF THE NIPPLE ARRAY AND THAT OF "QUARTER WAVE PLATE

Due to the difficulty in realizing a smooth transition between two medias, one of which normally is air and the other is glass, this principle has not been used very much in optics (however, see Strong 1950) The antireflection coating which nowadays is common on almost all lenses is made in another way It consists of a thin layer of a material whose index of refraction satisfies the equation

$$n_3 = \sqrt{n_1 \quad n_2}$$

where n is the index of refraction of the layer, n_1 is the index of refraction of the lens and n_2 that of air ($n = 1$) For a wavelength where this coating is a quarter wavelength thick the surface reflection will be zero This

reduction in surface reflection, however, diminishes very fast as the wave length gets higher or lower than the optimal The antireflection coating is normally adjusted to be most efficient for green light This is the reason why coated optics appear purple in reflected light (red and blue)

Fig 39 shows the calculated decrease in reflection caused by a quarter wave plate (curve A) together with the decrease in reflection caused by the nipple array with nipples placed close together (curve B) The thickness of the "quarter wavelength plate has been chosen to be equal to half the size of the nipples ($d/2$) which gives a maximum antireflection at the same wavelength as the nipple array It is seen that the gradual transition impedance transformer (nipple array) reduces the reflection more effectively over a broader band of wavelengths than does the quarter wave plate It should also be borne in mind that wherever the reflection is decreased the transmission is correspondingly increased, as has been demonstrated in our experiments with models (Figs 19 and 29) and is also evident from theoretical considerations

The theoretical calculations have been made for radiation at 0 degrees incidence (normal to the plane of the cornea) The reflection amplitude will increase as the angle of incidence decreases because the path length (apparent thickness of the coating or array) increases Because the path length changes as the cosine of the angle of incidence this makes little difference for values near 0° and becomes increasingly important near grazing incidence The quarter wavelength plate used in optics is fairly sensitive to angle of incidence because of the sharpness of its effect But for the gradual transition transformer such as the nipple array angle of incidence is less critical because the effect is relatively broad banded As illustrated by curve A on Fig 39 the quarter wave plate is most effective at 1/4 wavelength and is effective for an extremely narrow range of values of λ/d The simulated nipple array the calculations show is most effective at 1/2 wavelength (and in construction should therefore be in thickness about 1/2 wavelength of light in the middle of the visible spectrum) And because its effect is so broad banded, neither the thickness of the layer nor the angle of incidence of the radiation are very critical in comparison to the quarter wave plate



PART III

DISCUSSION

A THE ULTRAVIOLET REGION OF THE SPECTRUM

(Wavelengths shorter than 300 mμ)

The experiments with models have shown that for wavelengths of microwaves short in comparison with the nipple spacing the nipple array has a characteristic *effect on the pattern of energy transmitted by the model* The effect appears as a sharp increase in "optical density" as measured on the geometrical axis of the model and as a decreased "optical density" when measured off of the model's axis We interpret this to be the result of an interference phenomenon, a redistribution of the transmitted energy caused by the nipple array We did not confirm this phenomenon on actual corneas In our microwave experiments we found that the maximum observed effect shifted systematically towards the longer wavelengths with increasing dielectric constants Our experiments are not sufficiently precise in this matter for us to predict the exact extent and amplitude of the wave diffracted by the nipple array in actual corneas, but the indications are that this diffraction is greatest in the ultraviolet Interactions between the array and incoming light waves of the visible spectrum are of particular interest (see section II 2) but delineation of these interactions will have to *wait for further investigations*

Our spectrophotometric measurements of insect corneas demonstrate an increased density with a peak at 280 mμ This increased density has been shown to be a function of the thickness of the cornea and is independent of the presence of the nipple array It is probably due to the protein structure of the cornea

B THE VISIBLE SPECTRUM

(Wavelengths between 300 mμ and 700 mμ)

Experiments with models in which the microwavelength is long in comparison with the nipple constant show that the nipple array acts to decrease reflection from the front surface of the model and causes a concomitant increase in the amplitude of the transmitted wave These general observations hold over a very broad range from $\lambda/d = 1.5$ to $\lambda/d = 5.0$ *This should mean that for the actual cornea there should be a similar* effect extending between 300 mμ and 1 μ

Comparative spectrophotometric measurements performed on insects both with and without the corneal nipple array have confirmed the fact that reflection from the front surface of corneas with the nipple array reflects substantially *less light between the wavelengths of 366 mμ to 700 mμ* Direct confirmation of the increase in transmission would be difficult to obtain This is true both because the expected increase is small in propor-

tion to the intensity of the transmitted light and because in practice it would be difficult to provide adequate controls In this last respect, the microwave experiments have an advantage The nipples may be easily removed without substantial alteration to the model It should be noted that what alteration does occur consists of a shortening of the model In spite of the fact that the model is shorter without the nipples and that the microwaves must pass through less material, the amplitude of the transmitted wave is decreased when the nipples are removed

As shown in the preceding chapters the nipple array acts as an antireflection coating of the cornea and the decrease in reflection from the front surface is accompanied by a corresponding increase in transmission of light through the cornea

From the theoretical calculations it would be expected that the nipple array should reduce the intensity of reflection from the front surface of the cornea, and from the front surface of the model, by about 2 log units at the maximum when the nipples are placed close together (Fig 38 B) This theoretical figure is not achieved for the spectrophotometric measurements The measurements show only about 0 5 log units decrease in reflection As has been stated earlier, we were not satisfied that the backside reflection had been sufficiently reduced to permit truly accurate measurements of the front side reflection Because of this unavoidable backside reflection, our measurements of the front side reflection may be too high

Direct observation of the *living* light adapted night moth eye indicates that the backside reflection is lower than we were able to achieve by floating the corneas on india ink The reason for this may be that while the india ink absorbs the transmitted light, the reflection from the back surface is practically unabated because of the difference in refractive index of the cornea and the aqueous vehicle of the ink It is reasonable to assume that the reflection from the backside of the living light adapted moth cornea is much smaller because there is certainly no abrupt change in refractive index between the cornea, the balance of the dioptrics, and the pigment cells The pigment cells are analogous to the india ink, but the vehicle of the pigment in the living eye is matched in refractive index with the cornea It is therefore reasonable to assume that in the living light adapted night moth eye the reflection of visible light from the cornea approximates the theoretical values shown by curve B in Fig 38 which would mean that the reflection from the front surface of the insect cornea possessing the nipple array should be decreased about 2 log units in intensity, and the transmission of light is correspondingly increased in amplitude by about 0 02 log units

C BIOLOGICAL SIGNIFICANCE

From direct experimental evidence that has been confirmed and extended by the use of a mathematical model it has been shown that the corneal nipple array functions as an impedance transformer to decrease the reflection of light from the front surface of the cornea and correspondingly increase its transmission While the biological significance of this impedance transformer has not been verified by experiment, we deduce on the basis of behavioral observations and from closely related experimental evidence that the nipple array could give to the animals that possess it two important advantages under specified conditions (1) greater camouflage and (2) increased visibility

CAMOUFLAGE — The weight of evidence suggests that the nipple array is confined mostly to the nocturnal insects It is absent in many diurnal *Diptera* but present in the mosquito, which is largely nocturnal For the *Lepidoptera* though, it exists in both diurnal and nocturnal forms While the nocturnal moths are far more numerous than the diurnal butterflies in this order observation of the behavior of moths and butterflies indicates they both rely heavily on camouflage when they are inactive When the sun disappears behind a cloud the butterflies often become less active coming to rest on a tree trunk or foliage As they do this, the importance to them of expert camouflage is at once apparent Gone are the brilliant colors their striking allure shielded from the passersby s eyes by the drab and inconspicuous appearance of their folded wings Fig 40 illustrates the appearance of an active *Kalima* butterfly (to the left) and this same butterfly at rest (to the right) This dramatic change of appearance is a result of the fact that the upper wing surfaces, seen when the animal is active are brightly colored and indeed attract attention while the under wing surfaces seen when the animal is inactive present a skillfully camouflaged appearance This is true not only for most butterflies, but also for most moths There is however one part of the butterfly and of the moth that lies exposed and *apparently* uncamouflaged This is the eyes They are readily discerned in Fig 40 If the front surface of the corneas covering a large part of the head of these nocturnal insects were to reflect five per cent of incident sunlight it could be argued that these inactive animals would be easy prey to birds and other enemies that rely on acute vision for locating their food These *Lepidopteran* eyes though ordinarily appear to be almost black even in bright sunlight in the case of moths and they have a dull mat appearance in the case of butterflies This lusterless corneal appearance undoubtedly tends to blend with the natural setting of these animals and it is



Fig 40 Kalima butterfly as it would appear in activity (to the left) and at rest (to the right) (From the collection of the American Museum of Natural History New York)

therefore our hypothesis that for both the butterflies and the moths the corneal nipple array acts to enhance camouflage during the daylight hours For the moth, at least, the nipple array assists in camouflage only when the eye is light adapted When it is dark adapted, there is an intense reflection from the eye s tapetum (Leydig 1864) that is much greater than the reflection from the front surface of the cornea

VISIBILITY — In optics antireflection coatings have an important function in reducing multiple reflections Analogously, the nipple array reduces the intensity of multiple reflections (ghosts) within the eyes of insects For the insects any such advantage should be useful for diurnal and nocturnal animals

The advantage of the increased transmission of light caused by the nipples is less easy to gauge Since the increase is small it will probably be of less value for the diurnal forms possessing this advantage

In order to discuss the role played by the increased transmission for nocturnal animals the spectral quality of night light must be taken into account There is rather strong radiation in the infrared putatively ascribed to the effects of cosmic radiation on the upper atmosphere, and a

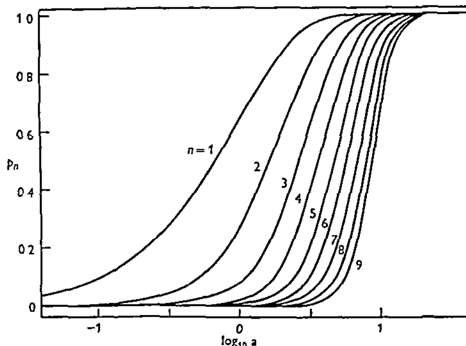


Fig 41 Poisson probability distributions For any number quanta per flash the ordinates give the probab lities (*Pn*) *that the flash will deliver to the retina n or more quanta as a* function of the mean number (*a*) of quanta in log units

spectral band in the visible range resulting from reflected sunlight From theoretical considerations we may state that the nipple array is relatively ineffectual in the infrared, and hence the animal can derive *no advan* tage in this regard

For the visible spectrum however there will be an increase in the in tensity of the transmitted light amounting to 5% of the incident intensity or 0 02 log units at the most A 5% increase in intensity seems trivial and is probably useless at high ambient light levels But because the *nipple* array is chiefly confined to nocturnal animals the arguments relating to a possible increase in visibility near absolute threshold should be carefully *reviewed These arguments are based* on the classical threshold experi ments of Hecht Schlaer and Pirenne (1942) Fig 41 shows theoretical curves reproduced from that paper The number of quanta in a series of short flashes is not constant but varies around a mean value and the proba bility that a flash contains a certain number of quanta is given by the Poisson distribution In Fig 41 *are graphed* Poisson distributions showing the probability for a flash containing *n* or more quanta (ordinate) as a function of the mean number *a* of quanta in log units (abscissa) In

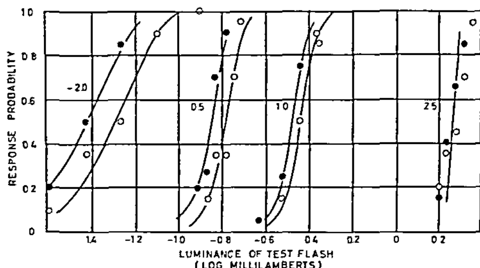


Fig 42 Near instantaneous thresholds as a function of level of light adaptation. Probability of seeing as a function of stimulus intensity for a circular stimulus area 30 min. in diameter presented to the fovea Figures beside curves indicate log intensity of adapting lights —2 0 log units was near absolute threshold for cones Solid and open circles represent experimental results for two subjects Curves are theoretical. Unpublished figure from experiments by C. G Mueller and L. R Wilcox of Columbia University (by permission) See text and Mueller and Wilcox, 1954

Hecht, Schlaer and Pirenne s experiments the actual measured values of the dark adapted human s probability of reporting he had seen a light flash (frequency-of-seeing curves) were found to fit the curves marked $n=5$, 6 and 7 for three different observers The light was presented to an extrafoveal area. The results were found to agree with theoretical calculations based on physical measurements of the probable amount of light reaching the receptor cells of the retina

The meaning of the curves of Fig 41 may be stated quite simply If the subject were able to see flashes with one or more quanta absorbed by the photopigment at threshold the slope of the curve showing the probability as a function of intensity of the light necessary to evoke a response covers a very broad range of intensities This range decreases and the curves get steeper when the threshold is assumed to correspond to a larger number of quanta absorbed Since the curves are then steeper, a relatively small change in the intensity of the flash will give a larger increase in the probability of seeing There is an indication from the work of Mueller and Wilcox (1954 that for moderately light adapted eyes these curves continue to follow the Poisson distribution and become even steeper up to a point Mueller and Wilcox studied the instantaneous threshold for a stimulus area located on the fovea The resulting frequency-of-seeing curves

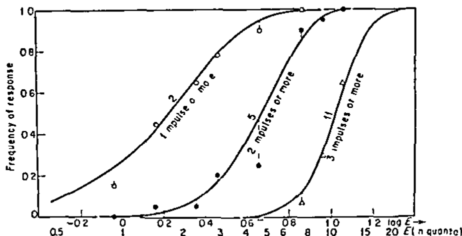


Fig 43 Thresholds to dim light flashes for single ommatidia of the compound eye of *Limulus* Single active optic nerve fiber preparation Abscissa intensity of flashes of light Ordinate proportion of responses containing one or more two or more or three or more nerve impulses Symbols experimental points Curves are theoretical Probability that a flash contains *n* or more quanta given to the left of each curve Experiments of Hartline Milne and Wagman (1947) Graph from Ratliff (1962)

are shown in Fig 42 (Mueller and Wilcox 1954) The numbers to the left of the curves are the relative intensities of adapting lights in log units The open and closed circles are measured values for two different subjects The solid lines are theoretical curves For adapting lights up to about 1000 times the absolute threshold of the cones the frequency-of seeing curves steepen according to the theory Even for the experiment in which the adapting light was 1 0 the measured values fall on the theoretical curves But for adapting intensities of more than three log units above the absolute threshold the measured values tend to fall away from the theoretical curves Thus for the 2 5 curve the slope for the measured values is lower than that for the theoretical curve For an adapting intensity of 1 0 an increase of intensity of 0 02 log units increases the probability of seeing 10—15% around 50 per cent probability Thus, for the moderately light adapted eye, a small increase in the intensity of illumination for objects viewed at threshold results in a greater increase in the probability of seeing

There is evidence that this data is not only valid for humans it is also applicable for some lower animals The probability of "seeing at threshold also follows the Poisson distribution in the eye of the horseshoe crab (Hartline, Milne and Wagman 1947) For the *Limulus* eye the criterion for seeing is the discharge of optic nerve impulses in response to the flash The frequency of seeing curves (Fig 43) then resemble the human data.

It is necessary to be cautious in applying these relationships to the insect eye The reason for this is the presence of secondary iris pigment cells in some nocturnal forms It is known that with increasing light intensity, the incoming light is reduced as a result of migration of this secondary iris pigment (see, *e g*, Bernhard, Ottosson 1960, 1964, Hoglund 1963) Such migration would tend to nullify the effect of the increased transmission of light resulting from the nipple array, but counteracting this pigment migration is the presence of the tapetum in these insects, which exhibits a metalic like reflection and hence gives the rhabdom a second pass at the illumination The resultant of such interactions is difficult to calculate, but the possibility of enhanced visibility still remains There is no evidence that pigment migration *per se* impedes the steepening of frequency of seeing curves as the intensity of illumination increases We know this because of the observations in *Limulus*, where there is intraretinular cell pigment migration that roughly parallels the course of increased sensitivity during dark adaptation (Miller 1958, Hoglund, *personal communication*)

Leaving aside the imponderables of pigment migration, we deduce from experiments of the probability of seeing at absolute threshold that a 5% increase in intensity will result in about a 5% increase in the probability of seeing It is not inconceivable that such an advantage could favor the selection of the nipple array, but it would seem probable that in animals such as moths and butterflies the camouflage factor is more important

For nocturnal animals that do not rely on camouflage it is more reasonable to ascribe the selection for the nipple array as being the result of increased visibility As far as we know the mosquito does not depend on camouflage to a significant degree These animals are small and during periods of inactivity they hide in nooks and crannies, behavior that is entirely different from that of the *Lepidopters* Here again, the arguments must be applied with caution, for it is known that pigment migration does occur in some mosquito eyes (Sato 1961)

Thus some species may benefit more by increased visibility at night others by greater camouflage by day Though it is difficult to pinpoint the sources of evolutionary pressure that developed and maintain the nipple array, we know from numerous examples that nature has a way of making the most out of small advantages Finally, it is certainly true that the nipple array makes possible the transfer through the cornea of almost the maximum intensity of light that is physically possible

SUMMARY

Structure

In *electronmicroscopic* studies (Part I) we found that the corneal surface of the facets of certain insect compound eyes is completely covered with congruent cone shaped protuberances These nipples are arranged in a more or less perfect hexagonal array Both the altitude of the nipples and their center to-center distance is about 200 mμ, and there is evidence that the circular sections of the bases of the nipples touch each other, *i e*, that the nipples are placed as close together as possible This nipple array has been found in different species of moths, butterflies netflies mosquitos, and caddisflies but not in species of bees, houseflies and dragonflies

In some insects (*Musca*) the corneas have a smooth front surface with a dense cortical layer containing a nippled substructure, the spaces be tween the nipples are completely filled in with corneal material

Function

The function of this nipple array was elucidated by microwave experi ments on scaled dielectric models and by spectrophotometric investiga tions on insect corneas The results were confirmed by the application of a mathematical model

In the *microwave experiments* (Part II B) on scaled dielectric models the center to-center distance of the nipples was chosen equal to the nipple amplitude (called nipple constant) It was shown that for radiation wave-lengths less than 1 5 times the nipple constant there occurs an interference phenomenon (peaking at a wavelength 0 5 times the nipple constant) that results in zones of greater and lesser intensities of transmitted energy and that for wavelengths longer than the nipple constant the nipples act to decrease reflection from the surface of the model and correspondingly increase transmission through the model A transposition of the scale to the dimensions of the cornea and the wavelengths of light leads to the conclusion that the nipple array decreases reflection from the corneal front surface and concomitantly increases transmission through the cornea for light of visible and near ultraviolet wavelengths

In the *spectrophotometric experiments* (Part II C) *on transmission of light* through moth corneas it was shown that the transparency of the cornea is relatively constant for wavelengths between 320 and 700 mμ and that under 320 mμ there is an increased density peaking at 280 mμ The increased density in the near ultraviolet was shown *not* to be related to the corneal nipple array but was found to be a property of the corneal chitin itself The results of spectrophotometric experiments on the reflec tion of light of the different wavelengths confirm those of the microwave

experiments leading to the conclusion that the nipple array reduces the reflection of visible light from the front surface of the cornea

The results of the microwave and spectrophotometric experiments showing that the nipple array decreases the reflection and increases the transmission of visible light over a broad wavelength range were confirmed by the use of a *mathematical model* (Part II D) Using a digital computer, calculations were made of the expected intensity of reflection from a surface consisting of a nipple array such as those used in the microwave experiments Good agreement between the experimental and calculated results was obtained Calculation of the decrease in reflection expected to be caused by an array with nipples placed as close together as possible predicts a maximum reduction of 2 log units at a wavelength twice the amplitude of the nipples The calculations also indicate that such a nipple array is more broadly effective than a 'quarter wave plate'

We conclude that the nipple array acts as an impedance transformer to match the characteristic impedance (inverse of refractive index) of air to that of the lens material (chitin) and thus functions to decrease reflection from the corneal front surface and concomitantly increase the transmission of light through the cornea over a broad wavelength range

The *biological significance* of the nipple array for camouflage and increased visibility was discussed (Part III)

ACKNOWLEDGEMENTS

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STUD[illegible]
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ORSTADIUS BOKTRYCKERI AB
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This summary is based on studies reported in the following papers

I The effect of exercise on the vascular bed of skeletal muscle Kjellmer I Acta physiol scand 1964 *62* 18—30

II Capillary permeability in skeletal muscle during rest and activity Arturson G and I Kjellmer Acta physiol scand *1964 62* 41—45

III On the competition between metabolic vasodilatation and neurogenic vasoconstriction in skeletal muscle Kjellmer I Acta physiol scand In press

IV Accumulation of fluid in exercising skeletal muscle Jacobsson S and I Kjellmer Acta physiol scand *1964 60 286—292*

V An indirect method for estimating tissue pressure with special reference to tissue pressure in muscle during exercise Kjellmer I Acta physiol scand 1964 *62* 31—40

VI The potassium ion as a vasodilator during muscular exercise Kjellmer I Acta physiol scand In press

The papers are referred to by their Roman numerals in the text

The English was checked by L. James Brown

Introduction

No other physiological activity imposes such a stress on the circulation as physical exercise In consequence the reactions of the circulatory apparatus to exercise have been extensively studied Interest has been focused mainly on two aspects of the problem *viz* the regulation of the cardiac output and the regulation of the blood flow through the actual muscle during exercise While some of the major mechanisms governing cardiac activity during muscular work have been elucidated, the underlying mechanisms of vasodilatation in exercising muscle is almost as obscure today as it was in 1880, when Gaskell tentatively attributed the hyperemia of exercise to chemical changes in the active muscles rather than to impulses from vasodilator nerves

Since then numerous hypotheses have been presented to explain the dilatation Gaskell himself for example suggested that the known increase in the acidity of the muscle tissue during exercise might be responsible for the hyperemia It has however since been repeatedly shown that changes of pH cannot play more than a subordinate role in the production of exercise hyperemia This was convincingly demonstrated by Gollwitzer-Meier (1950) who measured the pH of the venous effluent from the exercising gastrocnemius muscle of dogs and found that exercise initially lowered the pH but that this effect was only transient and during the most intense vasodilatation the pH rose above the control level Despite these refutations Gaskell s conception still survives in many textbooks of physiology

Not only the acid metabolites (which include CO and lactic acid), but also the effect of other possible causal substances or conditions have been discussed *e g* acetylcholine histamine oxygen lack adenosine compounds and bradykinin So far however cumulative evidence does not favour any one of these possibilities as the cause of the hyperemia of exercise (for recent reviews see Hilton 1962 and Barcroft 1963)

In 1941 Dawes suggested that potassium ions might be released during exercise from the intracellular to the extracellular space and act there by dilating the muscle vessels It would, however appear that no quantitative experimental analysis has ever been undertaken to check the validity of this hypothesis

Aim of present studies

The present studies were started in an attempt to evaluate the hypothesis that potassium ions play a role in vasodilatation during exercise The first step was to measure the potassium concentration of the venous outflow from the calf muscles of cats and estimate how much it rose during and after tetanic muscle contractions and to compare the vasodilatation occurring during this exercise with that induced by intra arterial infusions of isotonic potassium salts at a rate producing the same increase of the venous potassium concentration Judging from the results of these experiments, the potassium ions released during exercise could account for about one third of the hyperemia (Kjellmer 1960, 1961) However, in view of probable diffusion gradients between tissue fluid and venous blood this figure was regarded as fictitiously low

Further penetration of this problem required additional information on the detailed reactions of the muscle vascular bed during exercise Using a modification of a plethyhmographic technique described by Mellander (1960) the reactions of the resistance vessels and of the capacitance vessels to exercise were assessed simultaneously with the determination of the capillary filtration coefficient (CFC) as well as changes in the mean capillary pressure

The results of that study raised several other questions During exercise the CFC rose Was this rise due to an increase of capillary permeability or of capillary surface area available for exchange or a combination of both? Further, the results were obtained in sympathectomized muscles But evidence has been presented (III) that in many situations the sympathetic nerves are activated during exercise How would the pattern of vascular response be affected by superimposition of a sympathetic vasoconstrictor fibre stimulation on exercise? Lastly, during exercise capillary filtration occurred from the intravascular to the interstitial space What factors limit this loss of fluid from the circulating blood to the active muscles and how does the fluid return to the blood?

When these questions had been answered and the detailed vascular response to exercise had been charted it was possible to return to the point of departure, namely the problem of the role played by the potassium ions in the production of the hyperemia of exercise

The purpose of the present studies was thus to find answers to the following questions

1) How do the various parts of the vascular bed in a skeletal muscle react to muscular activity?

2) Does the capillary permeability increase in a skeletal muscle during exercise?

3) How does superimposed activation of the vasoconstrictor fibres affect the pattern of vascular response during exercise?

4) What factors limit the outward filtration during exercise? Does the tissue pressure increase during accumulation of fluid in the working muscle?

5) Are the potassium ions released from the intracellular space during muscle activity in concentrations high enough to account for the hyperemia? Does intra arterial infusion of a potassium solution produce the same reponse in each section of the vascular bed as does exercise?

Material and Methods

The experiments were performed on cats except in II, where dogs were used because evaluation of capillary permeability required sampling of fairly large amounts of pure muscle lymph

The methods used in each study have been described in Papers I—VI Here only some points of general interest will be mentioned

Anesthesia Anesthesia was induced with ether In the earlier experiments (some experiments in I and IV) a mixture of chloralose 50 mg/kg and urethane 100 mg/kg was used Studies in this laboratory however, showed that this type of anesthesia tended to produce metabolic acidosis a disadvantage not attending the use of chloralose or nembutal alone (Berglund, Nylen and Wallentin, personal communication) Therefore either chloralose 70 mg/kg or nembutal 30—35 mg/kg was used instead The reaction of the circulation to each of these three types of anesthesia appeared to be largely the same

Blood substitute Blood lost during the preparation was substituted by dextran-Tyrode solutions, in the earlier experiments 6 per cent Macrodex® (Pharmacia) and in most experiments a low molecular weight dextran in 10 per cent solution (Rheomacrodex® Pharmacia) which does not decrease the suspension stability of cat s blood (Eliasson and Samelius Broberg 1963) Dextran was also used as the priming solution in the tubing and drop chamber of the flowmeter

Preparation The preparation of the calf muscles the isolation of their blood flow and the plethysmographic technique used in all studies but II are described in detail in I

The method used enables simultaneous recording of the arterial in flow pressure, the venous outflow pressure, the blood flow and changes in tissue volume

The calf muscles were prepared in the following way The paw was removed at the ankle joint and the tendons of the calf muscles were fixed to the distal parts of the tibia-fibula An incision of the skin was made as high as possible around the entire circumference of the thigh The skin was then dissected free from the thigh muscles down to the level of the knee. This skin collar was used to seal the plethysmograph in which the

calf was afterwards enclosed The thigh muscles were divided by thermo-cautery just above the knee A small hole was drilled in the femur, whose bone marrow cavity was then plugged with cotton The large and the small saphenous veins were ligated All vessels running to and from the calf were ligated except the popliteal artery and vein

The preparation obtained consisted of muscle skin and bone in the proportions 3 1 1 (mean weights found in 10 cats) The main routes of circulation through the skin were, however closed by ligating the saphenous veins and the saphenous artery so that the circulation through the skin was practically shut off The circulation through the bone and bone marrow presumably contributes but little to the blood flow in the popliteal vessels Thus as far as the present investigation is concerned the blood flow recorded from the popliteal vein could be considered a fairly pure muscle blood flow and to represent the sole drainage of the calf muscles

In some experiments the blood flow through the calf was kept constant by a perfusion pump operating on the arterial inflow

Nerve stimulation The sciatic nerve which contains all motor fibres to the calf muscles, was left intact in III but was severed in the other studies The peripheral end was stimulated with electrical square wave pulses to produce an isometric muscle contraction (both ends of the calf muscles being fixed) This contraction was used to imitate exercise The terms muscle contractions and exercise have been used interchangeably in these papers since the isometric contractions induce the same local blood flow response as do comparable muscle contractions producing a true movement (*e g* Anrep and v Saalfeld 1935)

Periods of muscle contractions at a rate of 0 5 to 4/sec were used to produce various levels of steady state vasodilatation Higher rates of contractions proved to impede blood flow and were therefore not used (I)

The motor fibres were activated at stimulus intensities far below the threshold for the vasomotor fibres in the sciatic nerve (I)

The vasomotor fibres running to the calf were activated separately by stimulating the lumbar sympathetic chains at the level of L_4 — L in III In III the sciatic nerve was not severed since practically all sympathetic fibres supplying the calf muscles run in the sciatic nerve (Bowling *et al* 1953)

Recording The mean arterial pressure in the contralateral femoral artery was recorded with a mercury manometer Venous pressures were measured with a manometer filled with saline from the venous outflow

tubing Blood flow was diverted from the cannulated popliteal vein through a drop chamber filled with inert silicone oil and recorded with a photo electric drop counter operating an ordinate writer Changes in tissue volume were recorded from the water-filled plethysmograph with a small piston recorder

Isotope experiments In some experiments changes in regional blood volume were assessed separately, using a combination of continuous recording of volume changes and of blood borne radioactivity (as described by Åblad and Mellander 1963, but with slight modifications, III) The red cells were tagged with Cr^{51} and the γ activity from the calf was monitored with a scintillation detector, a scaler unit and a ratemeter This combined recording was used in III, where volume changes due to filtration and to changes in regional blood volume occurred to some extent simultaneously It was also used in control experiments to test in a more direct way some conclusions arrived at in I

Calculations of CFC and of changes in the mean capillary pressure The capillary filtration coefficient (CFC) expresses the flow of filtrate across the capillary membrane in 100 g of tissue per unit pressure change, ml/(min × 100 g × mm Hg) The CFC was determined by raising the venous outflow pressure abruptly by a known amount and measuring the ensuing rate of net outward filtration Once the CFC was known, it was also possible to deduce how much the mean capillary pressure had changed from the control conditions, where the volume was constant (the isovolumetric state) This was done by dividing the rate of filtration by the corresponding CFC value The comparatively small errors involved in the determination and the calculation of CFC and of changes in mean capillary pressure are discussed in I

Results and Discussion

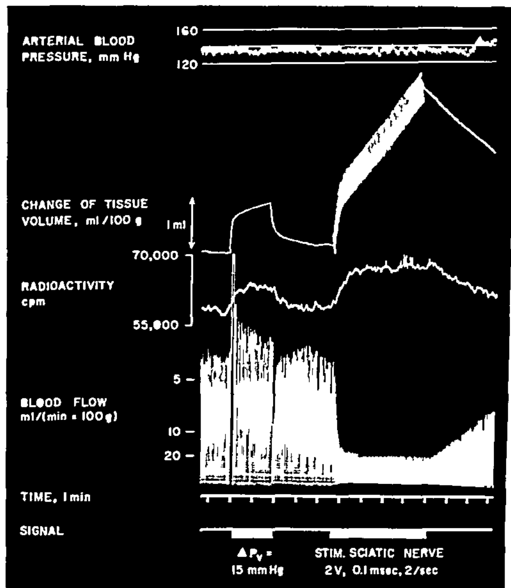
The effect of exercise on the various parts of the vascular bed in skeletal muscle

Muscle contractions induced a decrease of vascular resistance evidenced by an increased blood flow or — in the constant flow experiments — by a drop in the perfusion pressure

The exercise also produced an increase of tissue volume that occurred at two different rates *i e* a rapid phase followed by a slower one (Fig 1) The first phase coincided with the acceleration of the blood flow, while the second phase occurred when the blood flow had attained a stable high level The first phase was therefore attributed to an increase of regional blood volume, while the second phase was regarded as being independent of changes in regional blood volume (I) These conclusions have been fully corroborated by observations made in experiments using changes in radioactivity emitted from Cr^{51} labelled erythrocytes as a separate measure of changes in the regional blood volume Fig 1 shows a record obtained in such an experiment Note that the radioactivity increased during the phase of rapidly increasing volume but remained fairly steady during the second phase in spite of the fact that the increase in tissue volume during this phase was approximately three times as large as that noted for the rapid phase

The first phase of the volume record obtained during exercise could thus be used as a measure of the increased amount of blood contained in the capacitance vessels This increase of the regional blood volume was related to the degree of exercise — amounting to some 0 8 ml/100 g muscle during the periods of most severe exercise (I) Since resting cat skeletal muscle contains 2 5—3 ml blood/100 g (Klein 1945, Mellander 1960 p 38) the increase thus represented some 25—30 per cent of the resting regional blood volume

This increase may, theoretically, be due either to a decreased tone of the capacitance vessels allowing them to accommodate more blood at an unchanged distending pressure or to an increase of the distending trans mural pressure, *i e* either an active or a passive increase of the lumen of the capacitance vessels In an attempt to assess which of these two pos sibilities was more likely the experiments were repeated under constant



Cat 3 5 kg

Effect of exercise on blood pressure tissue volume radioactivity emitted from the erythrocytes in the calf and blood flow Before the exercise period the venous pressure was abruptly raised by 15 mm Hg to allow comparison between the change in tissue volume and radioactivity The radioactivity curve was photographically transposed from the original record Time constant of ratemeter 10 sec
Note that when exercise was induced the radioactivity (*i e* the regional blood volume) increased during the phase of rapidly increasing tissue volume while it stayed unchanged during the slower phase of increasing tissue volume

flow conditions (I) In such circumstances the decrease of perfusion pressure during dilatation prevented any extra distending pressure from reaching the postcapillary section, where the main capacitance vessels are localized This also prevented the increase of regional blood volume, which was taken as a sign that the change of regional blood volume normally occurring during exercise is merely a passive phenomenon

It might be argued that it is impossible to reveal an active diminution of tone in a denervated preparation where the capacitance vessels might already be maximally dilated However, the same technique has demonstrated that denervated capacitance vessels are not fully relaxed since they can respond to acetylcholine and adenosine triphosphate with a decrease of tone (Kjellmer and Odelram 1965)

The second slower phase of the volume increase during exercise which is not due to a further accumulation of blood in the vessels, must apparently be ascribed to a transcapillary transport of fluid into the extra vascular space of the muscles This loss of fluid from the blood has long been known (Ranke 1865) and has been studied mainly in terms of changes in hemoglobin concentration and plasma volume (see Holmgren 1956 ch 7) In the discussion of this displacement of fluid from the plasma to the active muscles two different mechanisms have been considered an increased tissue osmolarity because of the augmented muscle metabolism (*e g* Landis 1934) and an increased capillary pressure due to the vasodilatation (*e g* Barcroft and Kato 1915) The first process — a diffusion — is driven by a concentration gradient while the second process — a filtration — is caused by a hydrostatic pressure gradient Consequently a change in the hydrostatic pressure should alter the slope of the volume curve from that noted under control conditions if this slope were normally due to filtration but not if it were due to diffusion In order to ascertain which mechanism was at work the following type of experiment was performed (I) A period of exercise was induced at the normal mean arterial pressure This produced the usual biphasic increase of the tissue volume The mean arterial pressure was then lowered to half the control value by partial obstruction of the aorta After a few minutes the exercise was repeated but under these conditions of reduced transmural pressure (also at the capillary level) no increase in volume occurred during the slow phase Moreover the volume curve which usually sloped slightly downwards during the reduced pressure was not affected at all by superimposed exercise This excluded diffusion and at the same time indicated that the second slow phase of volume increase during muscle contractions was due to a net outward filtration

The interaction between metabolic and neurogenic control of the muscle vascular bed

It is generally accepted that the hyperemia of exercise is a local phenomenon, not induced by vasomotor nerves Thus sympathectomy of a limb does not affect the ability to increase the limb blood flow during and after muscle contractions (*e g* Grant 1938) But this does not preclude interaction between the vasomotor nerves and blood flow in exercising muscle Since there are two separate sets of fibres, muscle circulation can, theoretically, be interfered with in two ways

The cholinergic vasodilator fibres running to the skeletal muscles are not involved in homeostasis of the blood pressure So far these fibres have been found to be engaged only in a special hypothalamic pattern of discharge, which is so organized as to suggest an involvement in emergency situations (Eliasson *et al* 1951) Their actual role in the so called defence reaction has now been clearly demonstrated in a series of studies on cats (Abrahams, Hilton and Zbrozyna 1960, 1964) It remains to be shown whether these fibres, apart from such special situations, are also involved during exercise in general In an experimental investigation Dornhorst (1963) found no evidence of any activation of the vasodilator fibres during normal muscular work Though Blair, Glover and Roddie (1961) occasionally noted signs of slight activation of the dilator fibre supply to the arm muscles during leg exercise, such activation occurred only in inexperienced subjects and was not considered an integral part of the normal vasomotor response to exercise Moreover, the activation of these fibres does not increase the size of the functioning capillary surface area (Renkin and Rosell 1962), which implies that locally released metabolites are solely responsible for this important part of the dilatation during exercise It should further be noted that there is no need to postulate a nervous mechanism to explain the hyperemia of exercise, not even the initial dilatation since it has been demonstrated that the vasodilatation begins within one second of a very brief contraction and is unaffected by sympathetic block (Corcondilas Koroxenidis and Shepherd 1964) However, the borderline between normal exercise and situations involving mental stress where the dilator fibres are certainly activated (*e g* Barcroft *et al* 1960 Brod Hejl and Ulrych 1963), is of course diffuse, and in many situations a combination of the two patterns may occur

The role of the other fibre system, the sympathetic adrenergic outflow, during exercise has been elucidated in a number of studies The functional importance of sympathetic activation during exercise is demonstrated by the marked impairment of the circulatory apparatus during exercise after

total sympathetic block (*e g* Ashkar and Hamilton 1963) and by studies showing vasoconstriction in non-active regions (for references see III)

It was therefore assumed that the normal vascular response to exercise is often a result of the combined action of the metabolic factors and vasoconstrictor fibres Accordingly, the effects of sympathetic nerve stimulation were tested in resting and contracting skeletal muscles (III)

Sympathetic activation raised the flow resistance both in resting and contracting muscles to a degree that could be graded by the frequency of sympathetic stimulation Both in resting and exercising muscles the most intense sympathetic activation provoked a 4—6 fold increase of the flow resistance There was however one outstanding and consistant difference between the response during rest and activity namely that the vasoconstriction in resting muscles persisted practically unchanged throughout the period of sympathetic activation (up to some 10 min) while it vanished after a few minutes in the active muscles despite continued stimulation of the sympathetic nerves In other words, during exercise the resistance vessels are dominated by the metabolic factors which effectively prevent any unduly long throttling of the blood flow However the two sections of the resistance vessels reacted differently from one another the sympathetic constriction being maintained longer in the postcapillary resistance vessels and resulting in a moderate rise of the mean capillary pressure above the level observed during exercise alone

The CFC already high during exercise was either not affected by the sympathetic activation or increased further during the phase of vanishing constrictor response This together with the accentuated elevation of the mean capillary pressure raised the rate of outward filtration

The capacitance vessels responded to the sympathetic activation with constriction both during rest and exercise After elimination of passive pressure-dependent effects it was found that the increased tone of the capacitance vessels during sympathetic stimulation was maintained practically unaltered throughout the stimulation period both in resting and active muscles (III) This is compatible with the observations made both *in man (Merritt and Weissler 1959) and in dogs (Haddy et al 1954) that* venous tone increases in the innervated limb during exercise

There is thus a marked functional differentiation between the responses of the various consecutive sections of the muscle vascular bed when the limb is subjected to the two opposing influences of metabolic vasodilatation and neurogenic vasoconstriction The resulting pattern of the vascular response appears to combine two advantages the flow of blood through the muscle and the distribution of that blood within the muscle is regulated

by the metabolic demands of the muscle itself (except for short periods during increased nervous activity), while the amount of blood contained in the muscle is largely controlled by nervous constrictive factors which help to maintain an adequate venous return to the heart.

Mechanisms involved in the limitation of outward filtration during exercise

It was thus established that circulating fluid was transferred into exercising muscle by a filtration process The rate of outward filtration was related to the degree of vasodilatation and the severity of exercise and was as high as 0 75 ml/ (min × 100 g) at the highest work loads (I) Sympathetic activation superimposed on the muscle activity tended to increase the rate of net filtration still more

This loss of fluid can evidently not proceed indefinitely This raises the question What are the factors that limit the net outward filtration? On theoretical grounds two possibilities might be considered either fluid is transported back from the muscle to the circulation via the lymph at about the same rate as it is filtered or the filtration process must in some way be self limiting

Evaluation of the first possibility requires knowledge of the flow of lymph from the muscles during rest and activity Lymph was therefore sampled from the calf care being taken not do include skin lymph Lymph flow which was very low during rest increased during muscular activity but only enough to account for some 10 per cent of the capillary filtrate produced simultaneously In contrast virtually all proteins in the filtrate return to the blood via the lymph (Jacobsson and Kjellmer 1964)

The main part of the capillary filtrate formed during exercise thus accumulates in the muscle during the period of activity In order to evaluate possibility of limiting the filtration estimations were first made of the total amount of filtrate that could be accumulated per unit muscle mass during exercise When the period of muscular contractions was prolonged the rate of filtration gradually diminished and eventually after 15—30 minutes no net filtration occurred despite continued muscle activity After cessation of exercise the filtrate was gradually reabsorbed and the muscle finally recovered its original volume, but usually not until after at least one hour The filtrate content of the muscles which was sometimes as much as 10—15 per cent of the resting muscle weight, varied with the severity of exercise (IV)

Since the outward filtration was due to a rise in the hydrostatic capillary pressure the mechanism limiting filtration must be able to counterbalance the effect of this rise According to the well known Starling hypothesis this can be achieved by three different mechanisms an increase in the tissue pressure an increase in the plasma colloid osmotic pressure or a decrease in the tissue fluid colloid osmotic pressure Using the values

obtained for the total amount of fluid accumulated in the muscles it was calculated that, under the present experimental conditions, the plasma colloid osmotic pressure could not have increased by more than 1 mm Hg and that the tissue fluid colloid osmotic pressure had decreased by at most 2—3 mm Hg Thus the change in the gradient of colloid osmotic pressure may help to counteract the effect of the high capillary pressure, but a major portion of the increase of the capillary pressure (which was 17 mm Hg during maximum dilatation) must be counterbalanced by some other mechanism A rise in tissue pressure, therefore, was assumed to be the main limiting factor This conclusion was also supported by the production of some indirect evidence that swelling of the muscles is accompanied by an increase in the tissue pressure (IV)

It was, however, felt that the validity of this conclusion, which was based on indirect grounds only, should be checked experimentally by measuring the tissue pressure in the muscles during rest and at various degrees of swelling owing to accumulation of capillary filtrate

The literature was searched in vain for a satisfactory method for determining tissue pressure (V) A method was therefore worked out which, though cumbersome was believed to provide reliable information on tissue pressure in the calf muscles (V)

The principle of the method is as follows Veins are collapsible tubes therefore the pressure inside patent veins can never be lower than the tissue pressure If the pressure in the veins just outside the organ (here called central venous pressure — CVP) is lower than the tissue pressure, the pressure in the veins inside the organ (local venous pressure — LVP) is determined by the level of the tissue pressure and not by the CVP (*cf* Holt 1941 Ryder, Molle and Ferris 1941) Hence, the pressure in a vein may drop steeply at the exit of the vessel from an organ a drop known as the waterfall phenomenon (Permutt, Bromberger-Barnea and Bane 1963) This implies that a rise of CVP does not influence LVP until CVP has reached the prevailing tissue pressure Thus, the pressure level at which a gradual rise of CVP begins to increase LVP may be used to estimate the tissue pressure The plethysmograph can be used as a suitable means to determine this level since when the veins are almost completely collapsed, even very small changes of LVP are sufficient to produce readily discernible volume changes

The results obtained with this technique indicated that the pressure prevailing in the resting calf muscles was not quite uniform but that, as expected, the mean pressure was very close to that of the atmosphere When exercise was induced, tissue pressure increased and this increase

could be attributed to the accumulation of fluid in the muscles rather than to the contractions themselves The unevenness of the tissue pressures within the preparation then became very marked so that it was impossible to express the increase in a single figure, but it was quite obvious that the rise of tissue pressure during exercise was of the order of magnitude required to explain the cessation of outward filtration The previous indirect evidence (IV) for this conclusion was thus now supported by some more direct evidence (V)

The role of potassium ions in exercise hyperemia

After the preliminary studies on the relation between release of potassium ions and exercise hyperemia (Kjellmer 1960, 1961), it was thought that this work should be continued along two different lines

Firstly, the comparison between vasodilatation induced by potassium and by exercise was repeated under steady state conditions using a constant flow technique The constant flow was used partly because it proved easier to produce stable vasodilatation with potassium infusions than when blood flow varied, but mainly because any gradient of potassium concentration between interstitial fluid (from where it may be considered to exert its main action on the vascular smooth muscles) and venous blood (where it is measured) should increase at higher flow rates

Secondly, the response of the various consecutive sections of the muscle vascular bed to the potassium infusion was compared with that to exercise because if the potassium ions were responsible for a major part of the dilatation in active muscles the infusion of potassium ions should produce the same pattern of vascular response as that induced by muscle contractions

The potassium concentration in the venous outflow rose during exercise The rise was related to the work load and consequently to the degree of vasodilatation During exercise producing maximum dilatation the rise was about 100 per cent Intra-arterial infusion of isotonic potassium salts, even in low concentrations was followed by vasodilatation The dilatation produced by the potassium, which was expressed in relation to the corresponding exercise dilatation at each level of potassium concentration in the venous blood varied from about 25 per cent at the lowest, to about 65 per cent at the highest, degree of dilatation (VI)

Thus, 25 to 65 per cent of the hyperemia of exercise could be directly ascribed to the potassium ions released But there is reason to suppose the existence of a diffusion gradient for potassium under these conditions (VI) This would imply that the role of the potassium ions was greater than that estimated above Such underestimation should, for two reasons, be most marked at the lowest degrees of vasodilatation firstly the diffusion distances are longer in the resting muscles and shorten as the functioning capillary surface area increases, which it gradually does during increasing vasodilatation (I), and secondly a given gradient has its greatest effect when the vessels are not fully dilated, *i e* where the slope of the dose-response curve for the potassium dilatation is steepest.

The fact that the fraction of the dilatation attributable to the potassium ions was smallest when the dilatation was slight and increased at higher

degrees of dilatation is thus compatible with the existence of a concentration gradient It was therefore considered justified to conclude that the potassium ions probably accounted for as much as two thirds of the vasodilatation during muscular contractions

However, one difficulty remains, namely, the fact that, as a rule, intra-arterial infusions of potassium salts produced no maximal dilatations and that when the dose was increased the vessels did not respond with further dilatation but with constriction This reversal occurred when the potassium concentration in the venous blood had risen to 20—30 meq/lit (VI) Although such values are probably much higher than those met with under physiological conditions they nevertheless seem to present a potential danger to the muscle vessels namely to close them down during intense exercise However there is evidence that the constriction occurring in association with intra arterial infusion of potassium is limited to the larger arteries which may run outside the muscle tissue (Emanuel, Scott and Haddy 1959) Concordant herewith was the present finding that the CFC remained high or even increased further when the dose of potassium was raised sufficiently to produce constriction (VI) indicating that at least the precapillary sphincters and maybe also other small precapillary vessels within the muscle tissue remained dilated even when the doses of potassium infused were large This might also possibly explain why potassium infusions could not produce maximum dilatation

When the vascular pattern of response evoked by potassium infusion was compared with that produced by exercise it was found that both procedures induced a decrease of the vascular resistance an increase of CFC that was related to the degree of dilatation, but no change in the tone of the capacitance vessels and no signs of any increased capillary permeability in other words the two procedures produced identical patterns of response (VI) It should also be pointed out that this feature is by no means common to all dilating procedures — on the contrary the discriminating power of this kind of comparison has proved to be very high Acetylcholine adenosine triphosphate histamine and bradykinin have all been shown to produce response patterns differing from that of exercise in one respect or another (Kjellmer and Odelram 1965) So far potassium is the only compound tested that produces exactly the same response as exercise which supports indirectly the suggestion that potassium ions are reponsible for a major part of the hyperemia of exercise

Summary and Conclusions

The nature and cause of local circulatory adjustments to changes in muscular activity were studied with a plethysmographic technique, sometimes combined with isotope methods The method permits separate evaluation of the reactions of the resistance vessels, the capacitance vessels, the capillary filtration coefficient and the mean capillary pressure in the calf muscles of cats The method can also be used to estimate the tissue pressure in skeletal muscle The observations made appeared to warrant the following conclusions

1) Exercise reduces blood flow resistance, the reduction varying with the severity of the exercise The dilatation affects the precapillary resistance vessels relatively more than the postcapillary with a rise in the mean capillary pressure as a result The raised pressure head also reaches the capacitance vessels and distends them passively No active change of tone occurs in the capacitance vessels The raised capillary pressure leads to a net outward filtration which is facilitated by a simultaneous increase of the capillary filtration coefficient

2) The capillary permeability does not change during exercise The increased capillary filtration coefficient must therefore be ascribed to an opening up of precapillary sphincters with an increase in the surface area available for the exchange between blood and tissue

3) *When the vascular bed of skeletal muscle is subjected to the antagonistic* dilating influences due to exercise and to constricting influences due to sympathetic vasoconstrictor fibres, the various sections of the vasculature respond in a functionally differentiated way The resistance vessels and particularly the precapillary sphincters, are dominated by the vasodilative factors, while the capacitance vessels are more sensitive to the constrictive influence so that the blood flow to, and the distribution of blood within, the muscle is determined mainly by the metabolic requirements, while pooling of blood in the active muscle is prevented by nervous activity

4) The net filtration of fluid from blood to active muscles is many times larger than the lymph flow, which results in an accumulation of

fluid in contracting muscles The outward filtration is to some extent limited by changes in the gradient of colloid osmotic pressure across the capillary wall but the main limiting mechanism is the tissue pressure that rises gradually when the muscles are distended by capillary filtrate

5) The potassium ions released from the intracellular space during exercise reach such a high extracellular concentration that they can account for a major part of the vascular dilatation accompanying muscular activity When the diffusion gradient between the tissue and blood is taken into account, about two thirds of the hyperemia of exercise may be attributed to the potassium ions

The hypothesis that potassium ions are mainly responsible for the hyperemia of exercise receives indirect support from the fact that when given intra arterially potassium is the only compound hitherto known to produce a vascular response identical in every respect with that elicited by exercise

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BY

OLE SVENSMARK

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Contents

1 Nomenclature

The nomenclature of the *I U B Commission of Enzymes*[1] will be followed *Acetylcholinesterase* is the trivial name of acetylcholine acetylhydrolase (EC 3 1 1 7), formerly known as true cholinesterase (ChE) specific ChE e type ChE Type I ChE or aceto ChE *Cholinesterase* is the trivial name of acylcholine acylhydrolase (EC 3 1 1 8) formerly known as pseudo-cholin esterase unspecific ChE non specific ChE non specific acetyl ChE s type ChE type II ChE or Y ChE

In this review *cholinester hydrolase* is used to designate choline ester split ting hydrolases in general since cholinesterase formerly used in that sense is inconsistent with the new nomenclature

When necessary distinction will be made between *butyrylcholinesterases* and *propionylcholinesterases* according to the substrate split fastest in agree ment with a suggestion of *Richter* (1948)

For a discussion of the nomenclature of multiple forms of enzymes (iso-enzymes), see section 4 The nomenclature of genetically controlled cholin esterase variants is discussed by *Motulsky* (1964) and *Goedde* and *Baitsch* (1964) see section 4 2 1

The following abbreviations and names will be used ChE = cholinesterase AChE = acetylcholinesterase, DFP = diisopropyl fluorophosphate DEAE cellu lose = diethylaminoethyl cellulose *dibucaine* = *cincaine*[3] = 2 butoxy N (2 di ethylaminoethyl) quinoline 4 carboxyamide ASTRA 1397 = 10(1 diethylami nopropionyl) phenothiazine HCl ethopropazine[4] = 10 (2 diethylaminopro pyl) phenothiazine HCl

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[1]) *Report of the Commission on Enzymes of the International Union of Biochemistry I U B Symp Series no 20 Oxford Pergamon Press 1961*

[2]) United States Pharmacopeia.

[3]) World Health Organization

[4]) New and Non official Drugs

2 Introduction

Two main types of cholinester hydrolases are known (*Alles* and *H* 1940) (1) *Acetylcholinesterase* is present in nervous tissue, and in son and autonomic effectors for example in certain synapses and in the n endplate where it splits acetylcholine, the transmitter of nerve impu Similar enzymes occur in the electric organs of fish, here, in addition are considered essential for the production of electrical energy Enzyme the same type are found at the myotendinous junction, in certain s venoms in thrombocytes and red blood cells where their function is unkn (2) *Cholinesterase* occurs in certain ganglia, plasma glia, liver, intes muscle lung pancreas and other glands, its function is unknown Ch esterases are sometimes subdivided into butyryl and propionylcholinester according to which substrate is split fastest at optimal conditions S cholinesterases, which occur both in vertebrate and invertebrate plasma acetylcholine faster than they split other choline esters, and may be inclu among the acetylcholinesterases However the cholinester hydrolases repre a highly inhomogeneous group of enzymes and a rational classification nomenclature will have to await further studies

Cholinester hydrolases have been extensively studied and are the sub of more than 5000 articles The literature up to 1947 is covered by a rev by *Augustinsson* (1948) and the newer literature may be found in *Heff Handbuch der experimentellen Pharmakologie* (*Koelle* 1963 a) These other reviews and the subjects they deal with are listed in the Appen p 54–55

This review is concerned with the molecular properties of cholinester which hitherto have not been treated fully except in connection with studies of the structure of the active site of the enzyme Cholinesterases h not been isolated in a pure state and little is known about their chem and physical properties However indirect evidence as to their molecu properties may be derived from electrophoretic and chromatographic stu and from the effect of other substances on the properties of the enzy Most of these studies concern mammalian plasma cholinesterase only

concern cholinesterases from other body fluids and tissues and from other species

In spite of their interesting physiological function acetylcholinesterases are not dealt with here These enzymes are firmly bound to the cell structure and drastic treatments are required to render them soluble (*Lawler* 1964) It is still uncertain whether reproducible and homogeneous preparations can be obtained and the proteins may be altered during the procedure

The physiological function of cholinesterase is not known, there is however, no lack of speculation as to its role in the body The discovery that acetylcholinesterase and cholinesterase were different enzymes (*Alles* and *Hawes* 1940) led to the suggestion that cholinesterases are not concerned with nervous transmission (*Mendel* and *Rudney* 1943 1944 *Zeller* and *Bissegger* 1943) and the absence of distinct pharmacological effects in humans and animals after selective inhibition of cholinesterase is consistent with this view (*Comroe Todd* and *Koelle* 1946 *Hawkins* and *Gunter* 1946 *Koelle* and *Gilman* 1946 *Mazur* and *Bodansky* 1946) Neither does the correlation between the pharmacological effects and the degree of inhibition of acetylcholinesterase or the absence of correlation with the inhibition of cholinesterase indicate a physiological function of cholinesterase (*Blaschko Bülbring* and *Chou* 1949 *Hawkins* and *Mendel* 1949 *Shelley* 1955) In speculating about the function of cholinesterase one must remember that there are healthy individuals in whom the enzyme is absent probably due to silent genes see section 4 2 1 and 5 1

That the enzyme occurs in blood and almost all tissues might suggest that it functions by removing acetylcholine and other choline esters leaking out from nerve endings or produced in extraneural tissues (*Koppanyi* 1948 *Augustinsson* 1950) Thus γ aminobutyrylcholine is present in brain tissue (*Kewitz* 1959) and may be split by cholinesterase (*Holmstedt* and *Sjöqvist* 1960) The higher affinity of the enzyme for butyrylcholine than for acetylcholine led to the idea that the enzyme acts by inactivating butyrylcholine which might be synthetized by the choline acetylase system from butyryl coenzyme A formed in the course of fatty acid metabolism (*Clitherow Mitchard* and *Harper* 1963) However the rate of splitting of butyrylcholine is only about twice that of acetylcholine splitting and a specific removal of butyrylcholine by the action of cholinesterase seems hardly possible

The speculations also have been directed towards a possible role of the enzyme in the metabolism of choline (*Zeller* and *Bissegger* 1943) in myelination (for references see *Davies* 1954 1963) or in food assimilation (*Gerebtzoff* 1959)

Based on the finding of cholinesterase in certain ganglia, muscle and

secretory glands, the idea has anew been advanced that cholinesterase, as does acetylcholinesterase, plays a part in the transmission of nerve impulses. Selective inhibition of cholinesterase affects the motility of the intestines (*Comroe*, *Todd* and *Koelle* 1946, *Grob*, *Lilienthal* and *Harvey* 1947, *Koelle*, *Koelle* and *Friedenwald* 1950, *Burn*, *Kordik* and *Mole* 1952, *Jamieson* 1963), and the enzyme may participate in transmittory processes in other tissues as well (*Koelle* 1950, *Burn* and *Walker* 1954, *Häggquist* 1960; for reviews see *Koelle* and *Gilman* 1949, *Whittaker* 1951, *Burn* 1956, *Cullumbine* 1963, *Koelle* 1953 b).

3 Molecular Properties of Serum Cholinesterases

3 1 SOLUBILITY

Human and equine serum cholinesterases are highly soluble in water Precipitation does not occur after desalting of solutions of the purified enzymes on columns of Sephadex G 25 prepared with demineralized water of a conductivity of about 5 µmho (*Svensmark* unpublished) The solubility of human serum cholinesterase in 18 per cent ethanol in aqueous solution (ionic strength 0 02) has a minimum at pH 4 9 At higher ionic strength (0 1) in 18 per cent ethanol the solubility increases about 1000 times and has a minimum at pH 4 6 (*Surgenor et al* 1949) This indicates an isoelectric point at pH 4 6–4 9 which is higher than in aqueous solution (see section 3 3 1)

Human and equine serum cholinesterases are precipitated by 1 3 to 3 5 M ammonium sulphate and formerly the enzymes have often been classified as albumin like proteins (*Stedman Stedman* and *Eason* 1932 *Stedman* and *Stedman* 1935 *Faber* 1941 1943 b *Strelitz* 1944 *Augustinsson* 1944 1948 *Kalow* 1952) Curiously enough human and equine serum esterases (*Hanriot* 1896) which are most probably identical with cholinesterase were correctly classified as globulins by *Rona* and *Petow* (1924) *i e* a few years before the discovery that serum splits choline esters (*Engelhart* and *Loewi* 1930)

Human serum cholinesterase is precipitated at lower concentrations of ammonium sulphate than the corresponding enzyme from horse (*Rona* and *Petow* 1924 *Kalow* 1952) This difference may be due to co-precipitation, since the purified enzymes were precipitated by about the same concentration of ammonium sulphate (fifty per cent precipitation at 2 85 M for human ChE (purified 80 times) and at 2 70 M for equine ChE (purified 100 times) pH 7 22 C (*Svensmark* unpublished)

3 2 MOLECULAR WEIGHT

Human serum cholinesterase was purified 3400 times by fractional precipitation with ethanol in the presence of zinc ions (*Surgenor* and *Ellis* 1954) The preparation contained four protein fractions with the uncorrected sedimen

tation coefficients s_{20} = 5, 8, 10, and 14 S The 8 S fraction was the largest In the different steps of purification the cholinesterase activity was proportional to the amount of the 10 S fraction ($s^{0}_{20,w}$ = 12 S), suggesting that this fraction is identical with the enzyme From this assumption an enzyme concentration of 7 mg/l of human serum could be calculated Assuming a molecular weight of 300 000 for the 10 S fraction, the molar concentration of the enzyme is about 2 10^{-8} From kinetic studies with inhibitors *Goldstein* (1944) deduced a molar concentration of 5 10^{-7} in dog s plasma, and by a similar technique *Myers* (1952) found a concentration of 8 8 10^{-8} in horse plasma 7 9 10^{-8} in human plasma 5 8 10^{-8} in mouse plasma 4 6 10^{-8} in dog plasma, and 1 7 10^{-8} in rat plasma

Horse serum cholinesterase purified 14 000 times by fractional precipitation with ammonium sulphate, ultracentrifugation and electrophoresis, was practically homogeneous in electrophoresis at pH 8 5, in ultracentrifugal analysis one major peak contained the enzyme activity (s_{0}, uncorrected = 9 9 S) One mole of diisopropylfluorophosphate (DFP) combined with 84 000 g of the protein (*Jansz* and *Cohen* 1962) since one molecule of DFP combines specifically with one active site (see section 3 5 1 2), the findings suggest a maximum molecular weight of 84 000 per active site The sedimentation coefficient of the protein is not consistent with a molecular weight as low as 84 000 indicating that each molecule of the enzyme may contain 2–4 active sites

Human serum cholinesterase (*Stensmark* 1963 c) and equine serum cholinesterase (*Stensmark* and *Heilbronn* 1964) must have a molecular weight above 200 000 since they appear immediately after the void volume in gel filtration on Sephadex G 200 which retains molecules of molecular weights of up to 200 000

3 3 ELECTROPHORETIC PROPFRTIES

3 3 1 *Human serum cholinesterase*

A few years after the discovery of serum cholinesterase (*Engelhart* and *Loewi* 1930) an attempt was made to characterize and purify the enzyme by free electrophoresis (*Vahlquist* 1935) using the segmented U tube constructed by *Theorell* (1934) At pH 7 4 to 7 8 (ionic composition not stated) human serum cholinesterase migrated between albumin and globulin' By a similar technique *Glick* *Glauback* and *Moore* (1942) showed that the enzyme migrated together with α and β-globulins at pH 8 2 A partially purified preparation of human serum cholinesterase contained mainly α and β-globulins (*Cohn et al* 1946) and a highly purified preparation of serum cholin

esterase consisted mainly of α globulins (*Surgenor et al* 1949, *Kekwick Mackay* and *Martin* 1953 *Surgenor* and *Ellis* 1954) suggesting that the enzyme is an α globulin

In paper electrophoresis at pH 8 4–9 0 in barbital buffers the enzyme migrates between α_2 and β globulins (*Gregoire* and *Derrien* 1952 *Baker* and *Pellegrino* 1954, *Skorepa* 1956 *Pintér* 1957 *Cohen* and *Warringa* 1957 *Vincent* 1958 *Marton* and *Kalou* 1959 *Svensmark* 1961 a b see Fig 1 2)

The enzyme can be localized on the paper by the following methods

(1) by cutting the paper strip into narrow transversal sections eluting with water or buffer and determining the cholinesterase activity by conventional methods This procedure has the advantage that specific substrates or inhibitors can be used (*Gregoire* and *Derrien* 1952 *Baker* and *Pellegrino* 1954, *Skorepa* 1956 *Svensmark* 1961 b)

(2) Acid produced during the hydrolysis of substrate can be detected by means of acid base indicators (*Giri* and *Prasad* 1951, *Goutier* 1956 *Pokrousky* 1959)

(3) A number of histochemical methods have been adapted for use in paper electrophoresis (for references see *Burstone* 1962 *Koelle* 1963 b) Immersion of the paper strip in a solution of a naphthol ester and a suitable diazonium salt yields red spots at the site of cholinesterases (*Nachlas* and *Seligman* 1949 *Gomori* 1952 *Pintér* 1957) The method is sensitive simple and rapid but not specific since the substrates are split by other hydrolases such as acetylcholinesterases carboxylesterases (carboxylic ester hydrolase EC 3 1 1 1) and arylesterases (aryl ester hydrolase EC 3 1 1 2) The specificity can be somewhat improved by adding specific inhibitors *e g* eserine (*Pinter* 1957) the results are however rarely clear cut (*Paul* and *Fottrell* 1961 a *Ecobichon* and *Kalow* 1962)

(4) Substrates specific for cholinesterases were described by *Ravin Tsou* and *Seligman* (1951) β carbonaphtoxy choline and 6 bromo-2 carbonaphtoxy choline are split by the enzyme and β naphthol is spontaneously formed by decarboxylation. This product is coupled with a diazonium salt resulting in a reddish spot on the paper

(5) Indoxylacetate and some of its derivatives especially 5 bromoindoxyl acetate are split by acetylcholinesterases and cholinesterases and the indoxyl formed is oxidized in air into blue indigo (*Holt* 1952 *Holt* and *Withers* 1952)

(6) Similarly different esters of 2 6-dichlorophenol indophenol can be used (*Kramer* and *Gamson* 1958 *Master* 1960)

(7) The thiocholine method of *Koelle* and *Friedenwald* (1949) has been used with paper strips

(8) An attempt to use cholinesterase labelled with radioactive diisopropylfluorophosphate was not successful (*Cohen* and *Warringa* 1957)

Similar results were obtained in electrophoresis on starch or agar plates (*de Grouchy* 1958, *Dubbs Vivonia* and *Hilburn* 1960 *Paul* and *Fottrell* 1961 a *Thompson* and *Cook* 1961 *Harris Hopkinson* and *Robson* 1962, *Stern* and *Lewis* 1962, *Arfors Beckman* and *Lundin* 1963), and in column electrophoresis (*Augustinsson* 1958 a 1959 a c *Pilz* 1962) In column electrophoresis the enzyme is determined by conventional methods on the fractionally collected eluates Labelling of the enzyme with radioactive DFP has been used to trace the enzyme in column electrophoresis of human serum (*Cohen* and *Warringa* 1957) and of rabbit and guinea pig serum (*Goutier* 1956)

It thus seems well established that human serum cholinesterase is an α_2-globulin with slightly lower mobility than the main α_2-globulin fraction at pH 8 6 By changing the pH it is however, evident that the electrophoretic properties of the enzyme differ from those of the bulk of α globulins At pH 11 the enzyme behaves as a β-globulin, at pH 6 as an α_1-globulin, at pH 5 as an albumin, and at pH below 5 as pre albumin (Fig 1) This

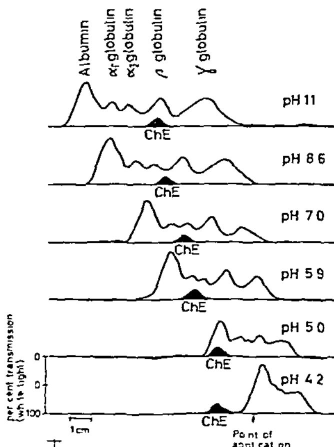


Fig 1

Paper electrophoresis of human serum at different pH Full lines protein concentration *Black areas* location of cholinesterase.

Apparatus LKB no 3276 with a constant-current power supply *Paper* Schleicher & Schüll no 2043 bmgl 40×425 mm, equilibrated for 3 hours in the apparatus before application of the sample. *Buffers* ionic strength 0 10 at pH 4 2–5 9 sodium acetate at pH 7 0–8 6 sodium acetate + barbital at pH 11 sodium glycinate *Conditions of the run* 1–4 C 1 0 mA per strip for 19 hours ± 10 min. *Samples* undialyzed human serum, 20 µl for the protein-strip 10 µl for the cholinesterase-strip *Staining* protein was stained with Amido Black 10 B cholinesterase was visualized with α naphthyl acetate and Fast Blue B *Scanning* After staining and drying of the strips they were scanned in a scanning device with a white light source and a linear output.

indicates that the enzyme is an acid protein with an isoelectric point below that of albumin (pH 4 7) The solubility of human serum cholinesterase in ethanol water indicated an isoelectric point of 4 6–4 9 (*Surgenor et al* 1949, see section 3 1), but later *Surgenor* and *Ellis* (1954) stated an isoelectric point of the enzyme of below 4 4 An estimate of the isoelectric point has been obtained in paper electrophoresis by determination of the electrophoretic mobility at pH 2 8 to 9 in phosphate acetate and acetate barbital buffers of ionic strength 0 08–0 1 (Fig 2) (*Svensmark* and *Kristensen* 1962 1963)

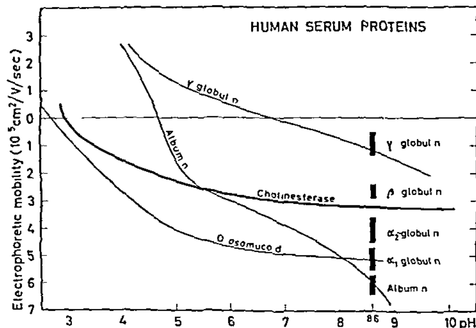


Fig 2

Electrophoretic mobilities of human serum proteins as a function of pH at 1 C
The mobilities of γ globulin and albumin were determined in paper electrophoresis in sodium acetate (pH 4 0–5 5) sodium acetate + barbital (pH 5 5–7 0) and sodium barbital buffer (pH 8 6–8 8) ionic strength 0 08–0 12 (*Waldmann Meyer* and *Schilling* 1959) The mobilities of cholinesterase and orosomucoid were determined in paper electrophoresis in sodium phosphate (pH 2 8) sodium acetate (pH 3 0–6 0) and sodium acetate + barbital (pH 6 0–9 8) ionic strength 0 08–0 10 (*Svensmark* and *Kristensen* 1963)

The migration of a substance in paper electrophoresis is affected not only by the electric field but also by electro-osmosis evaporation distortion of the migration path in the paper structure adsorption of protein to the paper and by changes in the electrolyte content of the paper during electrophoresis Using dextran as an uncharged reference substance *Schilling* and *Waldmann Meyer* (1959) and *Waldmann Meyer* and

Schilling (1959) corrected for these contributions to the migration and obtained free electrophoretic mobilities of human albumin and γ globulin identical with those obtained in free electrophoresis These methods were reviewed by *Waldmann Meyer* (1963)

With slight modifications of the procedure with respect to impregnation of the paper the concentration of dextran temperature and determination of the electric field *Svensmark* and *Kristensen* (1963) found mobilities of acid glycoproteins (orosomucoid coeruloplasmin) identical with those determined in free electrophoresis A drawback of the method is the slight change in ionic strength ($\Delta\mu = -0\,017$) and in pH when volatile buffers are used ($\Delta pH \approx \pm 0\,1$) The standard deviation of the determinations was 0 08 10^{-5} cm²/V/sec in 60 experiments

The electrophoretic mobilites of unpurified human serum cholinesterase and of preparations purified 285 and 5000 times were identical, indicating that protein protein interactions did not affect the mobility of the enzyme At pH 8 6 and 1° C the mobility was − 3 1 10^{-5} cm²/V/sec and the isoelectric point was at pH 2 9 − 3 0 For comparison the pH dependence of the mobility of human orosomucoid albumin and γ globulin are shown in Fig 2 For further discussion of the pH dependence of the electrophoretic mobility, see sections 3 4 3 and 3 5 2

3 3 2 *Equine serum cholinesterase*

Horse serum has often been preferred as a source of cholinesterase The enzymatic properties of equine cholinesterase are in several respects identical with those of the human enzyme but differences are observed with respect to substrate specificity (*Augustinsson* 1948, *Sturge* and *Whittaker* 1950, *Levy* 1951) inhibitor specificity (*Hawkins* and *Mendel* 1949, *Myers* 1952 *Austin* and *Berry* 1953 *Barstad* *Kramstad* and *Øksne* 1959) turnover number (*Berry* 1951) and reactivation and ageing after phosphorylation (*Heilbronn* 1963)

Several authors have determined the electrophoretic mobility of horse serum cholinesterase in segmented U tubes (*Theorell* 1934) *Glick* *Glauback* and *Moore* (1942) found that the enzyme is associated with α and β globulins at pH 7 5 The data of *Augustinsson* (1944) *and of Kraupp and Werner* (1947) are given in Fig 3 The conversion of the mobility at the experimental temperature (20° C) to 1° C was obtained by multiplying the mobility with η_0/η_1 where η_0 is the viscosity of water at 20° C and η_1 the viscosity of water at 1° C The migration of the enzyme was determined from measurements of the enzymatic activity in the different segments of the tube a procedure which gives rather inaccurate values of mobility Another drawback of the method is the asymmetrical migration in the anodic and cathodic compartments of the tube which is especially pronounced with high protein concentrations The experiments of *Augustinsson* (1944) were carried out

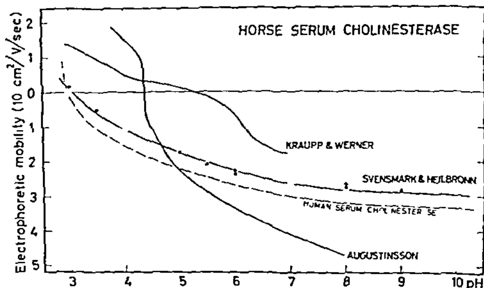


Fig 3

The electrophoretic mobility of horse serum cholinesterase as a function of pH at 1 C
The data of *Kraupp* and *Werner* (1947) were obtained in free electrophoresis (*Theorell* apparatus) in 0 2 M potassium phosphate buffers those of *Augustinsson* (1944) in the *Theorell* apparatus in sodium acetate buffers (pH 3 9–4 6) and sodium phosphate buffers (pH 4 8–7 8) ionic strength 0 1 and those of *Svensmark* and *Heilbronn* (1964) in paper electrophoresis in sodium acetate (pH 3–6) sodium acetate—sodium barbital (pH 6.5–9 0) and sodium glycinate buffer (pH 10) ionic strength 0 08–0 10 For comparison the mobility of human serum cholinesterase is indicated by the stippled curve (*Svensmark* and *Kristensen* 1963)

with 25 times purified preparations whereas those of *Kraupp* and *Werner* (1947) were made with only 4 times purified samples In the latter experiments the asymmetrical migration was pronounced and the measurements are unreliable (see also section 3 4 1)

The isoelectric point of equine serum cholinesterase was at pH 4 4 (*Augustinsson* 1944) and 5 2 (*Kraupp* and *Werner* 1947) An isoelectric point of about 4 3 was suggested by *Heilbronn* (1962 b) on the basis of the migration of a purified preparation of the enzyme in paper electrophoresis Errors due to electro-osmosis evaporation and adsorption of protein to the paper were not considered The mobility of horse serum cholinesterase at pH 7 8 (−4 5 10^{-5} cm²/V/sec at 1° C), in *Augustinsson*'s (1944) experiments is not consistent with paper electrophoretic studies which show a mobility at pH 8 6–9 0 between that of α and β-globulin, *i e* about −3 10^{-5} cm /V/sec (*Gregoire* and *Derrien* 1952 *Pinter* 1957 *Heilbronn* 1962 b) In one study pH was

not stated (*Togni* and *Meier* 1953) Similar results have been obtained in starch gel electrophoresis (*Paul* and *Fottrell* 1961 a) and in column electrophoresis (*Augustinsson* 1958 a, 1959 a c) In column electrophoresis at pH 8 4 the rate of migration of horse and dog serum cholinesterase was lower than that of the human enzyme (*Augustinsson* 1961)

The electrophoretic mobility of a purified preparation of horse serum cholinesterase (*Heilbronn* 1962 b) was determined at pH 3 to pH 10 (Fig 3) (*Svensmark* and *Heilbronn* 1964) using the method of *Svensmark* and *Kristensen* (1963) At pH 8 6 the mobility was −2 8 10⁻⁵ cm²/V/sec in agreement with the localization of the enzyme between α and β globulin in paper electrophoresis The mobility varied with pH in the same manner as for human serum cholinesterase, and the isoelectric point was at pH 3 We have no explanation for the difference between the results of *Augustinsson* (1944) (pH 4 4) and our results The buffers used at pH 3–4 6 were identical in the two studies, at higher pH *Augustinsson* used phosphate buffers whereas we used acetate barbital buffers The high value (pH 5 2) of the isoelectric point reported by *Kraupp* and *Werner* (1947) may be due to methodological artifacts (see pp 18–19 22)

3 3 3 *Serum cholinesterases of other vertebrates*

Relative electrophoretic migration has been determined at about pH 8 6 for serum cholinesterases in different vertebrates by column electrophoresis (*Augustinsson* 1958 a 1959 a b, c, 1960 b 1961 *Augustinsson* and *Olson* 1959) and by paper electrophoresis (*Goutier* 1956, *Pintér* 1957) (Table I) The plasma of mammals (except ruminants) and birds contain butyryl and propionylcholinesterases with mobilities of the same order as in human plasma The migration of the cholinesterases in horse plasma (*Augustinsson* 1961 *Svensmark* and *Heilbronn* 1964) and in dog plasma (*Augustinsson* 1961) was slightly slower than in human plasma An exception is the finding of cholinesterase activity in the γ globulin area in rabbit and guinea pig plasma (*Goutier* 1956) a finding not confirmed in later studies (*Augustinsson* 1958 a 1959 a c) In the case of rabbit, the disagreement may be explained by the fact that some individuals have two different cholinesterases (*Koelle* 1953)

The activity of the propionyl or acetylcholinesterases in ruminants and fish were too low to allow determination of their electrophoretic properties by column electrophoresis there is indirect evidence that the cholinesterase of goat plasma migrates in the α–β globulin area (*Augustinsson* 1959 a c) Observations on cholinesterase in amphibian and reptilian plasma are scarce The propionylcholinesterase of frog and the cholinesterase of turtle migrated

TABLE I

Electrophoretic mobilities and types of serum cholinesterases in vertebrates The electrophoretic mobilities are approximate they were estimated by comparison with the migration of human serum protein fractions at pH 8 4–8 6 in filter paper or cellulose powder electrophoresis The type of enzyme is indicated by the substrate split at the highest rate butyrylcholine (Bu) propionylcholine (Pr) or acetylcholine (Ac) The sera of animals marked with an asterisk contain more than one fraction of cholinesterase see ref 2 8

References 1 *Augustinsson* (1958a) 2 *Augustinsson* (1959a), 3 *Augustinsson* (1959b) 4 *Augustinsson* (1959c) 5 *Augustinsson* (1960b) 6 *Augustinsson* and *Olson* (1959), 7 *Goutier* (1956) 8 *Pintér* (1957)

Animal	Approximate electrophoretic mobility 10 cm V sec	Type of enzyme	References
Man	3 1	Bu	(see sect 3 3 1)
Monkey *(Macabus cynomolgus)*	3–4	Bu	2 4
Monkey *(Cercocebus torquatus)*	3–4	Bu	2 4
Dog*	3–4	Bu	1 2 4 5 8
Cat*	3–4	Bu	1 2 4 8
Horse	2 8	Bu	(see sect. 3 3 2)
Swine	3–4	Bu	1 2 4 6
Cow	?	Pr	1 2 4
Reindeer	?	Bu or Pr	2 4
Goat	(3–4)?	Ac	2 4
Sheep	?	Ac	2 4
Guinea pig	3–4	Bu	1 2 4
Guinea pig	1–2		7
Rabbit	3–4	Ac	1 2 4 8
Rabbit	1–2		7
Rat*	3–4	Pr	1 2 4 8
Cock and chicken	3–4	Pr	1 3 4
Duck	3–4	Pr	3 4
Turtle *(Testudo graeca)*	5–6	?	3 4
Frog *(Rana temporaria)*	5–6	Pr	1 3 4
Clawed frog *(Xenopus laevis)*	?	Bu	3 4
Pike *(Esox lucius)*	?	Ac	1 3 4
Cod *(Gadus callarias)*	?	Ac	1 3 4
Eel *(Anguilla anguilla)*	?	Ac	1, 3 4
Wrasse *(Labrus berggylta)*	?	Ac?	1 3 4
Frogfish *(Lophius piscatorius)*	?	Ac?	1 3 4
Sea scorpion *(Cottus scorpius)*	?	Ac	3 4
Mackerel *(Scomber scomber)*	?	Ac	3 4
Spiny dogfish shark *(Squalus acanthias)*	?	Ac	1 3 4

faster than the mammalian enzymes The esterase of the turtle exhibits unique properties in that it is sensitive to eserine but splits non choline esters at a higher rate than choline esters and it was suggested that this enzyme re presents a transition form between the carboxylesterases and cholinesterases (*Augustinsson* 1959 b, c)

Also electrophoresis on starch gel and agar plates has been used in com parative studies (*Lawrence*, *Melnick* and *Warner* 1960, *Paul* and *Fottrell* 1961 a, *Arfors Beckman* and *Lundin* 1963, *Hess et al* 1963, *Kaminski* and *Gajos* 1964, *Kaminski* and *Jeanne Rose* 1964 *Oki*, *Oliver* and *Funnell* 1964) However due to molecular sieving the migration in these media differed from that in paper and cellulose powder The resolution of the esterase fractions were higher than in paper and in addition to the main fractions of cholin esterase one or more weak bands were seen in sera from man and several other species (see section 4)

3 4 PROSTHETIC GROUPS OF SERUM CHOLINESTERASES

3 4 1 *Unknown groups*

The existence of a prosthetic group of unknown nature was proposed for equine serum cholinesterase by *Kraupp* and *Werner* (1947, 1948) and *Werner* and *Kraupp* (1948) They suggested an enzyme symplex" in the sense of Willstätter (*Willstätter* and *Rohdewald* 1931) in equilibrium with an active component (an agon) and a carrier protein (a "pheron) The dissocia tion of the symplex depended on the temperature, pH and dilution and the two components could be separated by electrophoresis The dissociation constant and ΔH of the reaction were calculated These conclusions were based on the asymmetrical migration of the enzyme in the cathodic and anodic compartments of the segmented U tube of *Theorell* (1934) (see sec tion 3 3 2) *Augustinsson* (1944) considered the asymmetry an artefact due to high concentrations of protein in the cell, and the conclusions of *Werner* and *Kraupp* (1948) seem less convincing also because they introduce obscure corrections for the effect of light pH and dilution on the enzyme

3 4 2 *Metals*

There is no evidence that serum cholinesterases are metalloproteins Desalt ing of the enzyme or the presence of chelating agents does not abolish the enzyme activity indicating that reversibly bound metal ions are not involved in the function of the enzyme Nor are there indications that metalloenzyme inhibitors interact with irreversibly bound metal The possibility remains that

the protein contains metal which is without importance for the function of the enzyme

It has been suggested that magnesium or calcium were required for the function of cholinesterases (*Ammon* 1943) Moderate inhibition of the enzyme was obtained by removal of these ions or by addition of fluoride oxalate or citrate The inhibition was however not complete even with high concentrations of the inhibitors (*Ammon* 1943 *Dybing* and *Loe* 1956) *Barnard* (1943) suggested that the enzyme comprises a haeme structure, and he found iron in various preparations of cholinesterases and acetylcholinesterases (*Barnard* 1946) These preparations were far from pure, and the indication of the haeme structure was only indirect in that cyanamide evoked a cholinergic response in animals Furthermore absence of an inhibitory action of cyanide and sodium azide indicates that iron is not involved in the enzymatic mechanism (*Ammon* 1943 *Mounter* and *Whittaker* 1953)

3 4 3 *Sialic acid*

A crystalline glycoprotein (mucoprotein) fraction of horse serum with cholinesterase activity was prepared by *Bader Schütz* and *Stacey* (1944, 1945) The specific activity of the preparation was only 20 times higher than in serum and *Mendel Rudney* and *Strelitz* (1944) assumed that the crystals contained at least 99 7 per cent impurities More convincing evidence that serum cholinesterase contains a carbohydrate moiety was presented by *Surgenor* and *Ellis* (1954) They purified human serum cholinesterase about 3400 times by precipitation with cold ethanol in the presence of zinc ions The preparation contained 11 per cent carbohydrate indicating that the enzyme is a glycoprotein The preparation was, however far from pure and the possibility remained that the carbohydrate was associated with the impurities

Direct analysis of possible carbohydrate components of the enzyme has to await the preparation of the pure enzyme It has however, been possible to show by indirect means that sialic acid is a component of human serum cholinesterase (*Svensmark* 1961 a b 1963 a *Svensmark* and *Kristensen* 1962 1963) Treatment of serum or purified preparations of the enzyme with neuraminidase (*N acetylneuraminate glycohydrolase EC 3 2 1 18*) changes the electrophoretic mobility significantly without abolishing the enzymatic activity of the protein At pH 8 6 the native enzyme migrates between α and β-globulin and the neuraminidase treated enzyme together with γ globulin (*Svensmark* 1961 a b) The change in mobility of the enzyme upon treatment with neuraminidase was also seen in column electrophoresis of a highly purified preparation of the enzyme (*Augustinsson* and *Ekedahl* 1962) and in starch gel electrophoresis of normal sera (*Harris Hopkinson* and

Robson 1962, *Ecobichon* and *Kalow* 1963 a) An estimate of the isoelectric point of the neuraminidase treated enzyme was obtained by determining the electrophoretic mobility at pH 2 8–9 in paper electrophoresis (*Svensmark* and *Kristensen* 1962 1963) with corrections for electro-osmosis, evaporation and adsorption of protein to the paper (see section 3 3 1) The mobility pH curve was about parallel to that of the native enzyme (Fig 4) At pH 4 to 9

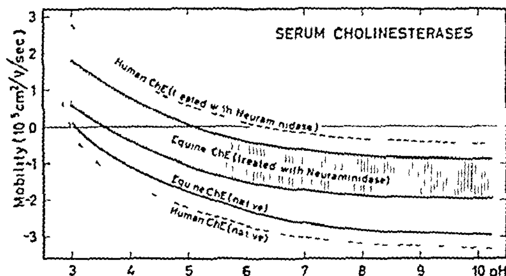


Fig 4

Electrophoretic mobilities as a function of pH at 1 C of human and equine serum cholinesterases before and after treatment with neuraminidase

The mobility was determined in paper electrophoresis in sodium phosphate (pH 2.8) sodium acetate (pH 3–5 5) sodium acetate + barbital (pH 6–9) and sodium glycinate buffer (pH 10) ionic strength 0 02–0 10 (*Svensmark* and *Kristensen* 1963 *Svensmark* and *Heilbronn* 1964) After treatment with neuraminidase equine serum cholinesterase appears on the paper as an elongated spot corresponding to the range of mobilities indicated by the cross-hatched area

the mobility was about 2 9 units higher than for the native enzyme and at pH 3 this difference decreased to about 2 units The isoelectric point was at pH 6 7–7 0 That the curves were parallel from pH 4 to 9 indicates that neuraminidase does not change the polypeptide part of the molecule The decrease of the slope at pH 3 is consistent with the assumption that sialic acid residues (pK 2 6) are removed in such a way that charged sites are not exposed The exact number of sialic acid residues cannot be determined but the gradual decrease of the electrophoretic mobility during incubation with neuraminidase indicates that several (e g more than five) sialic acid residues

are involved A small number of residues should give rise to a distinct step-wise decrease in mobility (*Svensmark* 1961 b *Ecobichon* and *Kalow* 1963 a)

In chromatography on DEAE-cellulose the native enzyme appears together with acid proteins after albumin whereas the sialic acid free enzyme appears together with β- and γ globulins (*Svensmark* 1961 b)

In experiments with 500 times purified preparations the neuraminidase treated enzyme was half precipitated at 2 4 M ammonium sulphate and the native enzyme at 2 7 M (pH 7 0 22 C) Both forms of the enzyme appear immediately after the void volume in gel filtration on Sephadex G 200 After treatment with neuraminidase the purified cholinesterase is less stable in dilute solution than the native enzyme thus it is inactivated faster by standing at room temperature and by electrophoresis on cellu lose columns (*Svensmark* unpublished)

The enzymatic properties of the sialic acid free serum cholinesterase are identical with those of the native enzyme with respect to splitting of acetyl choline and butyrylcholine (*Svensmark* 1961 b) propionylcholine and to inhibition with neostigmine, and to the Michaelis constant with acetylcholine as substrate (*Augustinsson* and *Ekedahl* 1962) Dibucaine and fluoride in hibited the two forms of the enzyme alike (*Ecobichon* and *Kalow* 1963 a)

Horse serum cholinesterase is also a sialo-protein as indicated by the change in electrophoretic mobility induced by the treatment with neuraminidase (*Augustinsson* and *Ekedahl* 1962 *Heilbronn* 1962 a) A 1200 times purified preparation of the enzyme contained 3 2 per cent sialic acid (*Heilbronn* 1962 b) The neuraminidase treated horse serum cholinesterase is however, not homogeneous in paper electrophoresis The enzyme appeared as protracted spots indicating that sialic acid free enzyme occurs with a spectrum of differ ent mobilities By the procedure of *Svensmark* and *Kristensen* (1963) the electrophoretic mobilities of the neuraminidase treated enzyme was deter mined at pH 3 to 10 (Fig 4) (*Svensmark* and *Heilbronn* 1964) The mobil ity of neuraminidase treated horse serum cholinesterase is lower than that of sialic acid free human serum cholinesterase at all pH values and the isoelectric point is at pH 3 6–5 2 suggesting a more or less complete removal of sialic acid residues from a single molecular form of the enzyme

As with human serum cholinesterase the stability decreases after the treat ment with neuraminidase The enzymatic properties are not changed by the removal of sialic acid residues

3.5 SIDE CHAIN GROUPS

3.5.1 *Functional groups*

3.5.1.1 *The active site* Information on the side chain groups has been obtained by indirect methods especially as to the functional groups involved in the catalytic function of the enzyme These groups are confined to a small area of the protein molecule the active site (*Haldane* 1930 *Koshland* 1960) The structure of the active site has been investigated by (1) studies of the kinetics of the hydrolysis of different substrates, and of the effect of different inhibitors (2) studies of the effect of agents which interact with different side chain groups (3) analysis of peptides obtained by degradation of cholinesterases which have been labelled at or near the active site by radio active organophosphorus compounds *e g* diisopropylfluorophospate ($DF^{32}P$) and (4) experiments with esterase models *i e* compounds which accelerate the hydrolytic splitting of esters

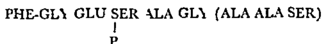
The amino acid composition of the active site and the mechanism of hydrolysis are treated in several recent reviews (*Nachmansohn* and *Wilson* 1951 *Whittaker* 1951 *Wilson* 1954 1955 1960 *Bergmann* 1955 1958 *Cohen et al* 1955 1959 *Hartley* 1955 *Nachmansohn* 1955 1959 1962 *Barnard* and *Stein* 1958 *Davies* and *Green* 1958 *Bender* 1960 *Koshland* 1960 *O Brien* 1960 *Cohen* and *Oosterbaan* 1963 *Jansz et al* 1963 *Sanger* 1963) Problems concerning the active sites of similar hydrolases *e g* chymotrypsin and trypsin have been discussed by *Neurath* *Dixon* and *Pechere* (1958) *Koshland* (1960) *Hartley* (1963) *Keil* (1963) *Neurath* (1963) and *Orekhovich et al* (1963) The section below deals solely with the amino acid composition of the active site

One of the features which distinguishes the cholinesterases from non-cholin esterases (arylesterases carboxylesterases) is their affinity for cationic substrates and inhibitors This was attributed by *Zeller* and *Bissegger* (1943) to *the presence of two different sites in acetylcholinesterase an anionic and an esteratic site* Cholinesterases were assumed to have only an esteratic site which could explain that they are not inhibited by high concentrations of substrate as is acetylcholinesterase *Bergmann* (1958) on the other hand suggested that anionic sites are involved in the active site of both types of cholinester hydrolases one in cholinesterases and two in acetylcholinesterases The problem is still controversial (see the reviews mentioned above and the discussions by *Augustinsson* 1960 1963)

3.5.1.2 *Serine* Direct evidence of the side chain groups involved in the active site of cholinesterases has been obtained by analysis of the amino acids

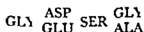
and peptides arising from the degradation of cholinesterase preparations labelled with DF³²P and similar compounds In such experiments labelled O serine phosphate was invariably found in the digest (*Cohen Oosterbaan* and *Warringa* 1955 *Cohen et al* 1955, *Jansz Brons* and *Warringa* 1959) The same results were obtained in experiments with acetylcholinesterases (*Schaffer, May* and *Summerson* 1954 *Cohen Oosterbaan* and *Warringa* 1955 *Cohen et al* 1955) chymotrypsin (*Schaffer May* and *Summerson* 1953 *Oosterbaan et al* 1958 a b, *Cohen et al* 1959) trypsin (*Cohen et al* 1955) carboxylesterase (*Cohen et al* 1955 *Dixon Go* and *Neurath* 1956 *Oosterbaan Jansz* and *Cohen* 1956 *Jansz Posthumus* and *Cohen* 1959 a b), thrombin (*Gladner* and *Laki* 1957) subtilisin (*Matsubara* 1959), elastase (*Naughton et al* 1960) acid phosphatase (*Greenberg* and *Nachmansohn* 1962) and in alkaline phosphatase (*Milstein* 1964 *Zwaig* and *Milstein* 1964)

By sequence analysis of a ³²P labelled peptide from degraded horse serum cholinesterase the following amino acid sequence was found (*Jansz Brons* and *Warringa* 1959)



where PHE = phenylalanine GLY = glycine GLU = glutamic acid SER = serine and ALA = alanine The sequence of the amino acids in brackets was not determined

The sequence is similar to that in other hydrolytic enzymes reacting with DFP In acetylcholinesterase alkaline phosphatase carboxylesterase chymotrypsin elastase thrombin and trypsin the sequence was



where the group adjacent serine may be either aspartic or glutamic acid and the other group glycine or alanine (for references see *Cohen* and *Oosterbaan* 1963 *Jansz et al* 1963 *Sanger* 1963) In proteinases from *Bacillus subtilis* and *Aspergillus oryzae* also phosphorylated by DFP the sequence was threonine—serine P—methionine—alanine (*Sanger* and *Shaw* 1960 *Shaw* 1963) Certain phospho-enzymes contain serine in their active site in phosphorylase *a* neighbouring amino acids were isoleucine and valine (*Fisher et al* 1959) and in phosphoglucomutase alanine and histidine (*Milstein* and *Sanger* 1961)

In the different hydrolases investigated the diisopropyl phosphoryl group is attached at the oxygen of the hydroxyl group of serine (*Cohen et al* 1959)

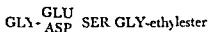
Upon storage (ageing) of a DFP treated sample of cholinesterase or acetylcholinesterase an increasing amount of the inhibitor was found as the monoisopropyl phosphoryl group, and the amount of the diisopropyl phosphoryl group decreased correspondingly The amount of the diisopropyl phosphoryl groups corresponded to the amount of enzyme which could be reactivated by nucleophilic agents such as hydroxamic acids or oximes (*Berends et al* 1959 *Cohen et al* 1959, *Jansz Brons* and *Warringa* 1959, *Jansz et al* 1953) Isopropanol is set free during this process (*Cohen et al* 1959), and it may be assumed that the monoisopropyl phosphoryl groups is attached to the enzyme by two bindings Similar results have been obtained with chymotrypsin (*Lee* and *Turnbull* 1958) These reactions may represent the chemical basis of the ageing phenomenon i e the occurrence with time of phosphorylated enzyme complexes which cannot be reactivated (*Hobbiger* 1955, 1956 *Jansdorf et al* 1955 *Wilson* 1955, and *Davies* and *Green* 1956)

The finding of phosphorylated serine residues in the digests of inhibited enzymes does not prove that this amino acid is involved in the active site of the enzyme DFP may be bound near the active site in such a way that it presents a steric hindrance for the combination of substrate and enzyme or the diisopropyl phosphoryl group may be bound at another site and then migrate to serine during the degradation procedure (*Schaffer May* and *Summerson* 1953 1954 *Hobbiger* 1955, *Jansdorf et al* 1955 *Mounter et al* 1957) It is even possible that the serine residue arises from impurities rather than from the enzyme though this does not seem likely since some of the hydrolases investigated were used as nearly pure preparations

That serine is essential in the hydrolytic catalysis is indicated by the isolation of an intermediate acetyl-enzyme-complex (*Balls* and *Aldrich* 1955 *Balls* and *Wood* 1956 *Oosterbaan* and *van Adrichem* 1958 *Spencer* and *Sturtevant* 1959 and by finding of acetylated serine in peptides obtained by degradation of such complexes (*Jansz Berends* and *Oosterbaan* 1959)

The problem of the active site of esterases has been studied in experiments with esterase models i e compounds which catalyze the hydrolytic splitting of esters (for references see *Westheimer* 1953) In this connection the observation by *Langerbeck* and *Baltes* (1934) is of interest that benzoylcarbinol and other alcohols accelerate the splitting of esters Serine itself does not catalyze ester splitting (*Rosengart* 1960) nor does it combine with organophosphorus compounds as do esterases (*Wagner Jauregg* and *Hackley* 1953, *Ashbolt* and *Rydon* 1957 But when the aliphatic hydroxyl group is labilized by hydrogen bonding to an O or S atom in the molecule, as in β-dimethyl aminoethanol N-oxide or β-hydroxyethyl sulfoxide it accelerates the hydrolysis of esters considerably *Schatzle Rottenberg* and *Thürkauf* 1959 *Jakoblet*

and *Rosengart* 1961) The reactivity of serine in the enzyme molecule may be affected by similar bonds to adjacent groups *e g* the unprotonated nitrogen of imidazole (*Brouwer* 1957 *Cunningham* 1957, *Westheimer* 1957) It has been suggested (*Rydon* 1958) that serine occurs in the enzyme as a cyclic compound (Δ-oxazoline) which combines readily with DFP (*Porter, Rydon* and *Schofield* 1958) When included in a peptide, however the reactivity with DFP diminishes (*Hanson* and *Rydon* 1962) Nor does N acetyl serineamide exhibit notable nucleophilic properties (*Bruice et al* 1962) For further discussions of the reactivity of serine see *Jansz et al* (1963) and *Westheimer* (1963) Future results with modified serine hydroxyl groups may be anticipated, especially with the newly synthetized tetrapeptides (*Kienhuis et al* 1961 *van de Linde et al* 1961)



Hitherto however attempts have failed to phosphorylate such peptides by DFP (*Kienhuis* and *van de Linde* 1963)

3 5 1 3 *Histidine* Though direct evidence is lacking certain findings point to imidazole groups as components of the active site of cholinesterase and acetylcholinesterase The pH dependence of the cholinesterase activity with an optimum of about pH 8 indicates that a basic group (pK 6–7) and an acid group (pK 9–10) play a role in the active site of cholinesterases and acetylcholinesterases (*Wilson* and *Bergmann* 1950 a, b *Wilson* 1954 *Bergmann et al* 1956 *Shukuya* and *Shinoda* 1956, *Mounter et al* 1957) Neither of these pK values points to serine but the pK of 6–7 for the basic group is consistent with the presence of imidazole Similarly the pH dependence of the inhibitory effect of organophosphorus agents indicates that a group with a pK of about 6 6 is essential for hydrolysis (*Mounter et al* 1957) The ionization heat (6 5–8 5 kcal/mole) of cholinester hydrolases (*Shukuya* and *Shinoda* 1956) *is identical with that of imidazole It is consistent with these* findings that agents which combine with imidazole inhibit the action of cholinester hydrolases Cholinesterase as well as acetylcholinesterase are inhibited by dinitrofluoro benzene and ethyl bis (β-chloroethyl) amine and acetylcholinesterase by diazobenzene sulfonic acid (*Mounter et al* 1957) These agents are however not specific for imidazole groups in that they also attack amino groups cysteine tyrosine and tryptophan (for references see *Boyland* 1948 *Barnard* and *Stein* 1958) Experiments on the pH dependence of enzyme activity and on the heat of ionization of the basic functional groups have

been interpreted to indicate amino groups as functional groups (*Hase* 1952 a, see section 3 5 1 7)

The ageing phenomenon involved in the inhibition of cholinester hydrolases by diisopropylfluorophosphate (see section 3 5 1 2) has been taken as evidence that the phosphoryl group migrates from histidine to serine (*Schaffer May* and *Summerson* 1953, 1954, *Hobbiger* 1955, *Jandorf et al* 1955, *Mounter et al* 1957) That a diisopropyl phosphorylated enzyme can shift to a monoisopropyl phosphorylated enzyme makes this assumption unlikely (for references see *Cohen* and *Oosterbaan* 1963)

Imidazole has also been assumed to be part of the active site in chymotrypsin and trypsin In chymotrypsin, inhibition by selective photooxidation of imidazole groups indicates a functional role of these groups (for references see *Jandorf et al* 1955 *Cohen et al* 1959, *Cohen* and *Oosterbaan* 1963) Histidine has furthermore been found in labelled peptides obtained by degradation of chymotrypsin inhibited by a radioactive organophosphorus compound (*Schaffer et al* 1956) On the other hand, enzymatically active peptides obtained from trypsinogen did not contain histidine (*Visuanatha* and *Liener* 1960)

Imidazole functions as an esterase model in that it catalyzes the hydrolysis of p-nitrophenyl acetate (*Hartley* 1955 *Bruice* and *Schmir* 1956 1957 *Bender* and *Turnquest* 1957 a, b, *Brechner* and *Balls* 1957) Histidine and histamine too accelerate the splitting of esters (*Gero* and *Withrow* 1957, *Rosengart* 1960) Imidazole combines with organophosphorus compounds but the complexes are easily hydrolyzed (*Wagner Jauregg* and *Hackley* 1953) Although the evidence is only indirect that imidazole is a component of the active site of cholinesterases the amount of indirect evidence is overwhelming and it may be justified to include imidazole in cholinester hydrolase models (*Bergmann* 1958 *Krupka* and *Laidler* 1961 a b c d, e)

3 5 1 4 *Tyrosine* The pH dependence of substrate hydrolysis and the effect of inhibitors (see section 3 5 1 3) indicate an acid group with a pK of 9–10 in cholinester hydrolases thus may be the hydroxyl group of tyrosine as suggested by *Wilson* and *Bergmann* (1950 a) and *Bergmann* (1958) Where as tyrosine combines readily with organophosphorus compounds (*Ashbolt* and *Rydon* 1952 1957) it accelerates the splitting of esters only weakly (*Rosengart* 1960) Phenols and polyphenols as well as combining with organophosphorus compounds (*Augustinsson* 1952, *Jandorf et al* 1952 *Berry* 1953 *Berry et al* 1955) catalyze the splitting of esters (*Rosengart* 1960 *Rosengart* and *Shepshelevich* 1962) The ultraviolet spectrum of purified liver esterase changes when the esterase is allowed to react with tetraethylpyrophosphate

and the change has been interpreted as indicating phosphorylation of the hydroxyl groups of tyrosine (*Ro engart* and *Kartasheva* 1959)

3 5 1 5 *Carboxyl groups* The presence of acid groups at both anionic and the esteratic site has been proposed (*Wilson* and *Bergmann* 1950 a *Bergmann* 1958) That glutamic acid is adjacent to serine (see section 3 5 1 2) is consistent with this assumption Though there is no direct evidence, this carboxyl groups has been assumed to be a part of the esteratic site (*Krupka* and *Laidler* 1961 a, b c, d, e) for further discussion of the role of carboxyl groups in esterases see *Cohen et al* (1959)

The inhibition of cholinesterase by zinc ions is consistent with a functional role of carboxyl groups since zinc ions combine with these groups (*Perkins* 1961 1964) rather than with histidine (*Gurd* and *Goodman* 1952) Horse serum cholinesterase is inhibited by 22 per cent at a zinc concentration of 1 7 10^{-4} M at pH 8 0 with acetyl choline as substrate (*Frommel Herschberg* and *Piquet* 1944) With human serum cholinesterase and benzoylcholine as substrate (10^{-4} M) at pH 8 5 in 10 mM TRIS buff r a 50 per cent inhibition was obtained in about 5 10^{-6} M zinc acetate The inhibition decreased with increasing H^+ and Na^+ concentrations (*Stensmark* unpublished)

3 5 1 6 *Amino groups* From the pK and the heat of ionization of the basic group in the active site of cholinesterases and acetylcholinesterases *Hase* (1952 a) suggested that amino groups rather than imidazole were involved (see section 3 5 1 3) The inhibition of the enzyme activity by amino group reagents such as formaldehyde and nitrous acid was cited in support of this view (*Hase* 1952 b) However to inhibit cholinesterase activity completely formaldehyde must be in high concentration (above 0 1–0 2 M) (*Bernheim* and *Bernheim* 1936 *Corteggiani et al* 1939 *White* and *Winternitz* 1939, *Hardwick* and *Palmer* 1961) The inhibition of a purified preparation of human serum cholinesterase is progressive, and increases with increasing pH (*Hase* 1952 b)

Lysine accelerates the splitting of tributyrin (*Gero* and *Withrow* 1957) and of p nitrophenyl acetate (*Ro engart* 1960), but it does not combine with organophosphorus compounds (*Berry et al* 1955) Lysine has been found in DFP labelled peptides obtained by degradation of trypsin (*Dixon Go* and *Neurath* 1956 *Dixon Kauffman* and *Neurath* 1958 a b), but not in cholin ester hydrolases

3 5 1 7 *Sulfhydryl groups* Cholinester hydrolases were previously assumed to be sulfhydryl enzymes in that their catalytic function was supposed to de pend on the presence of intact SH groups in the enzyme molecule (*Nach mansohn* and *Lederer* 1939 *Barron* and *Singer* 1943 *Stadie Riggs* and *Hau*

gaard 1955 *Gordon* and *Quastel* 1948 *Thompson* 1948 *Hase* 1952 b) This conclusion was based on the inhibitory effect on the enzyme of oxidizing and mercaptide forming reagents (for references see *Markwardt* 1953, *Mounter* and *Whittaker* 1953) Not all SH reagents were inhibitory, and with others the inhibition was only slight The most pronounced inhibition was obtained with different arsenicals it was, however competitive and easily reversed by dilution or dialysis, indicating that mercaptides were not formed. Therefore, the general assumption nowadays is that cholinester hydrolases are not SH-enzymes (*Goldstein* and *Doherty* 1951 b, *Markwardt* 1953 *Mounter* and *Whittaker* 1953 *Hargreave* 1955)

3.5.2 *Non functional groups*

The nature of the groups not directly involved in the catalytic function of the enzyme has been investigated indirectly by electrophoretic studies The increase in mobility of both human and equine serum cholinesterase after treatment with neuraminidase shows that the enzymes contain large numbers of neuraminic acid residues (see section 3.4.3) The pH-dependence of the electrophoretic mobility of the enzymes (Fig 4) indicates the presence of carboxylic groups (pK 3–5) and imidazole groups (pK 5.5–7), whereas α amino groups (pK 7–8.5), ε amino groups, SH and tyrosine-OH groups (pK 9–11.5) are relatively few or absent (*Stensmark* and *Kristensen* 1963) The positive charge of the enzymes is probably provided by arginine residues (pK about 12)

Using the change of mobility of human serum cholinesterase as an indicator of structural changes, attempts have been made to modify the protein without disturbance of its catalytic function (*Stensmark* unpublished) Blocking of the amino groups with formaldehyde acetylation with acetic anhydride, or succinylation of ε amino groups according to the procedure of *Klotz* and *Keresztes-Nagy* (1962) decreased the mobility of the enzyme indicating the presence of lysine residues

4 Multiple Forms of Serum Cholinesterases

In recent years enzymes in different forms or variants have been found in the same organ or body fluid in the same species. This enzyme heterogeneity may be due to the occurrence of truly different species of native proteins, of precursors or degradation products, or of proteins modified *in vivo* or in the preparatory procedure. It may imply the occurrence of (1) molecularly different species of protein with identical enzymatic properties, (2) proteins with apparently identical molecular properties and slightly diverging enzymatic properties, or (3) proteins with different molecular properties and slightly diverging enzymatic properties. Identity of enzymatic or molecular properties may, however, be difficult to establish with certainty.

Enzyme heterogeneity presents problems of nomenclature (see e.g. *Markert* and *Møller* 1959, *Furness* 1961, *Thompson* 1962, *Webb* 1964). The term *isoenzymes* and the somewhat slangy word isozymes have been used rather uncritically to designate such families of enzymes; when the term is not precisely defined it may be misleading.

Cholinesterases, too, occur in various forms differing both in serum and in tissues (see section 5.7) with respect to enzymatic and molecular properties. Certain variants are known to be genetically determined, others may be due to the occurrence of precursors and of degradation products.

Hence the description of the molecular properties of serum cholinesterases (section 3) may concern a mixture of proteins with nearly identical properties. However, one type predominates quantitatively, and the presence of small amounts of deviant types can not significantly affect the results.

4.1 VARIANTS WITH DIFFERENT MOLECULAR PROPERTIES

Surgenor and *Ellis* (1954) investigated whether there occurred more than one cholinesterase in human serum but found no evidence of heterogeneity (see also *Goldstein* and *Doherty* 1951 a). With the introduction of chromatographic and electrophoretic separation procedures, however, the heterogeneity of cholinesterases became obvious. Purified human serum cholinesterase appeared as two fractions in chromatography on calcium phosphate and the ion

exchange resin Dowex 2 (*Malmström Levin* and *Boman* 1956) and horse serum cholinesterase was separated into two fractions in DEAE cellulose chromatography (*Heilbronn* 1962 b)

In chromatography on DEAE-cellulose and DEAE Sephadex I have found three large and two small fractions of human serum cholinesterase The large fractions seemed identical in all respects and it is possible that they are due to co-elution of the same enzyme with different large peaks of inert protein The two small fractions may represent true cholinesterase variants (*Stenmark* unpublished)

In electrophoresis on columns of cellulose bimodal patterns were obtained with sera from cat and dog (*Augustinsson* 1959 a c 1961) and horse (*Heilbronn* 1962 b)

Findings in electrophoresis on starch gel are contradictory *Hunter Denuce* and *Strachan* (1961) *Thompson* and *Cook* (1961) and *Ecobichon* and *Kalow* (1962) found one spot of human serum cholinesterase, others found in addition one or more weak spots (*de Grouchy* 1958 *Dubbs Vivonia* and *Hilburn* 1960 *Lawrence Melnick* and *Weimer* 1960 *Bernsohn Barron* and *Hess* 1961 *Paul* and *Fottrell* 1961 a *Harris Hopkinson* and *Robson* 1962, *Stern* and *Lewis* 1962 *Harris et al* 1963 *Hess et al* 1963) The studies by *Harris* and his collegues are of special interest they used two dimensional electrophoresis on filter paper in one direction and on starch gel in the other Four cholinesterase components (C_1 C_2, C_3 C_4) of fresh human serum migrated between α and β globulin in filter paper electrophoresis C with a slightly higher rate of migration than the others They were distinctly separated in starch gel electrophoresis C_1 migrating fastest and the main component C_4 at the lowest rate All four fractions were sialo proteins the mobilities being reduced by neuraminidase (*Harris Hopkinson* and *Robson* 1962) In gel filtration on Sephadex G 200 the main fraction C_4 appeared immediately after the void volume then C_3 C and C_1 indicating decreasing molecular weights in that order (*Harris* and *Robson* 1963 a)

After storage for more than 10 days two additional spots appeared in two-dimensional electrophoresis and fresh foetal or cord blood had one additional spot *Harris Hopkinson* and *Robson* 1962) In about 5 per cent of the subjects an eighth component C_5 occurred which migrated both in paper and in starch gel at a lower rate than the other components In uni dimensional starch gel electrophoresis at pH 6 C_5 could be separated from C_4 Though C_5 is genetically determined it differed from the known inheritable cholinesterase variants (see section 4 2 1) (*Harris Hopkinson* and *Robson* 1962 *Harris et al* 1963)

Also in other vertebrate sera starch gel electrophoresis has shown multiple esterase patterns (*Lawrence Melnick* and *Weimer* 1960 *Bernsohn Barron*

and *Hess* 1961, *Hunter* and *Strachan* 1961 *Hunter, Denuce* and *Strachan* 1961, *Paul* and *Fottrell* 1961 a, b *Hess et al* 1963 *Kaminski* and *Gajos* 1964 *Oki Oliver* and *Funnell* 1964) Other esterases than cholinesterases may however, be involved as the methods used for visualizing the enzymes are not specific

In a few subjects the serum cholinesterase occurred with unusual electrophoretic mobilities In a woman (and possibly in her newborn infant) *Augustinsson* and *Brody* (1962) observed an unusually high mobility of the enzyme in that it migrated together with α_1 globulin at pH 8 4 instead of between α and β-globulin as in normal sera In a pool of sera from neurological patients the enzyme migrated together with γ globulin at pH 8 6 (*Svensmark* 1961 a b) The low mobility was due to the presence of neuraminidase (see section 3 4 3) which does not occur in normal serum

4 2 VARIANTS WITH DIFFERENT ENZYMATIC PROPERTIES

4 2 1 *Genetically controlled variants*

The finding of a genetically controlled atypical human serum cholinesterase with enzymatic properties diverging from the usual enzyme has attracted much interest in recent years and a number of reviews have appeared (*Kalow* 1959 a b 1962 *Fincham* 1960 *Augustinsson* 1961, 1963, *Harris* 1961 1962 *Lehmann Silk* and *Liddell* 1961 *Goedde Altland* and *Bross* 1963 *Lehmann* and *Liddell* 1964)

The discovery may be ascribed to the clinical importance of the enzyme variant When succinylcholine is used as a muscle relaxant certain patients with low serum cholinesterase develop prolonged apnoea (*Bourne Collier* and *Somers* 1952, *Evans et al* 1952 a, b 1953 *Reid* and *Neill* 1952 *Calvert et al* 1954 *Borders et al* 1955 *Foldes et al* 1955 *Kalow* 1956 a *Kalow* and *Gunn* 1957 *Lehmann* and *Simmons* 1958 *Kaufman Lehmann* and *Silk* 1960) The occurrence of unusually low enzyme activities could not always be explained by liver diseases or other pathological conditions (*Bourne* 1953 *Forbat Lehmann* and *Silk* 1953 *Lehmann* and *Ryan* 1956 *Lehmann* and *Silk* 1961) and it was found that the low activity occurred familialy (*Forbat Lehmann* and *Silk* 1953 *Allott* and *Thompson* 1956 *Kalow* 1956 b, *Lehmann* and *Ryan* 1956 *Lehmann Patston* and *Ryan* 1958 *Kaufman Lehmann* and *Silk* 1960) It was suggested that the variation of the enzyme activity was genetically determined by a pair of allelic genes so that homozygotes for the abnormal gene exhibited low values of enzyme activity, the homozygotes for the normal gene had normal values and heterozygotes had intermediate

values (*Allott* and *Thompson* 1956 *Kalow* 1956 b, *Lehmann* and *Ryan* 1956, *Lehmann, Patston* and *Ryan* 1958, *Kaufman Lehmann* and *Silk* 1960) Later studies have confirmed this scheme of inheritance and shown that the variation in activity was due to the presence of two different types of enzyme (*Kalow* 1956 a *Kalow Genest* and *Staron* 1956 b, *Kalow* and *Genest* 1957, *Kalow* and *Staron* 1957, *Kalow* and *Gunn* 1958/59) The abnormal homozygotes contained an "atypical" cholinesterase unlike the normal in substrate and inhibitor specificity (*Kalow* and *Lindsay* 1956 *Kalow*, *Genest* and *Staron* 1956 *Kalow* and *Davies* 1958 *Harris* and *Whittaker* 1959 1962 b, *Kalow* 1959 a, 1960, *Davies, Marton* and *Kalow* 1960), which is assumed to be catalytically less active than the normal enzyme (*Kalow* 1959 a, *Harris et al* 1960) Dibucaine (*Kalow* and *Lindsay* 1955, *Kalow, Genest* and *Staron* 1956 *Kalow* and *Genest* 1957, *Steinitz Eichhorn* and *Zelmanowski* 1963), a neostigmine analogue (*Kalow* 1962, *Harris* and *Robson* 1963 b), sodium fluoride and sodium chloride (*Harris* and *Whittaker* 1961 1963) inhibit the two forms of cholinesterase differently, and have been used to identify the different genotypes The incidence of heterozygotes in Canadian and European populations has been estimated at 3-4 per cent, and the incidence of atypical homozygotes at 1 in 3000–10 000 (*Kalow* 1956 b *Lehmann* and *Ryan* 1956, *Kalow* and *Staron* 1957 *Kalow* and *Gunn* 1958/59, *Kattamis et al* 1962 *Goedde* and *Altland* 1963) The same incidence was found in 104 Australian aborigines (*Horsfall Lehmann* and *Davies* 1963) and in the population of the island Tristan da Cunha (*Harris et al* 1963)

There is evidence that the type of cholinesterase in human serum depends on more than two allelic genes When serum cholinesterase was inhibited with fluoride instead of with dibucaine the same three genotypes were found, as well as three additional subgroups and family studies indicated the occurrence of another inheritable type of cholinesterase (*Harris* 1961, *Harris* and *Whittaker* 1961, 1962 a *Liddell Lehmann* and *Davies* 1963) A "silent" gene may be the explanation of anomalous findings of low or absent serum cholinesterase activity in certain families (*Kalow* and *Staron* 1957 *Harris et al* 1960 *Lehmann et al* 1960, *Liddell Lehmann* and *Silk* 1962, *Lehmann et al* 1963 *Goedde et al* 1964 *Motulsky* 1964) Thus four allelic genes have been suggested (*Lehmann et al* 1963) a normal gene (N), an "atypical" or dibucaine resistant gene (D) a fluoride resistant gene (F), and a silent gene (S) which could occur in the following combinations (the combination FS has not as yet been observed)

Homozygotes	NN	DD	FF	SS		
Heterozygotes	ND	NF	NS	DF	DS	FS

Recently however, another inheritable cholinesterase variant *Cs* was found (*Harris Hopkinson* and *Robson* 1962 see section 4 1) Its hereditary transmission can not be explained by simple allelism between the genes affecting the type of cholinesterase and evidence has been presented that two loci affect the cholinesterase production (*Harris Robson* and *Glen Bott* 1963) The incidence of the Cs variant in a British population was 5–10 per cent it was absent in the population of Tristan da Cunha (*Harris Robson* and *Glen Bott* 1963 *Harris et al* 1963), and in Australian aborigines (*Horsfall, Lehmann* and *Davies* 1963) A similar variant was found in equine plasma (*Oki Oliver* and *Funnell* 1964) The different cholinesterase variants their nomenclature and their incidence have been discussed by *Motulsky* (1964) and *Goedde* and *Baitsch* (1964)

4 2 2 *Molecular properties of atypical cholinesterase*

The different substrate and inhibitor specificity of normal and atypical human serum cholinesterase indicate a different structure of the active site of the enzymes That the difference is most pronounced with positively charged inhibitors points to different anionic sites (*Kalow* and *Davies* 1958 *Davies Marton* and *Kalow* 1960) The differential inhibition of the two enzymes by fluoride and high concentrations of chloride is not necessarily inconsistent with this view (*Harris* and *Whittaker* 1963)

Controversial results have been reported with respect to the electrophoretic and chromatographic behavior of the two enzymes *Kalow* (1959 a) did not obtain any separation by fractional precipitation with ammonium sulphate or by chromatography on DEAE-cellulose, and attempts to separate the two enzymes by electrophoresis were also unsuccessful (*Kalow* 1959 a, *Ecobichon* and *Kalow* 1963 a *Svensmark* 1963 a) On the other hand complete separation of the enzymes by paper electrophoresis at pH 9 7 and by chromatography on DEAE-cellulose was reported by *Liddell et al* (1962) Similarly a partial separation by paper electrophoresis of the dibucaine resistant and the fluoride resistant enzymes has been reported (*Liddell Lehmann* and *Davies* 1963)

The atypical enzyme is a sialo-protein as is the normal enzyme in that its rate of migration was reduced by treatment with neuraminidase (*Ecobichon* and *Kalow* 1963 a *Svensmark* 1963 a) The electrophoretic mobilities of native and neuraminidase treated atypical cholinesterase were identical with those of the normal enzyme (*Svensmark* 1963 a) as determined in paper electrophoresis according to the procedure of *Svensmark* and *Kristensen* (1963)

-2.3 Other variants

Inhomogeneity or individual variation of human serum cholinesterase is indicated by the greatly varying Michaelis constant of the hydrolysis of benzoylcholine (see *Kalow, Genest* and *Staron* 1956 a). From a bimodal form of the curve relating activity and substrate concentration, using butyrylcholine, *Heilbronn* (1958) concluded that two different cholinesterases are present in normal serum. Similar results were obtained with benzoylcholine and acetylcholine (*Berry* 1960). The substrate optima were differently affected by dibucaine and ethopropazine, and the results suggest that there are at least three enzymes involved.

5 Molecular Properties of Cholinesterases in Tissues, Spinal Fluid, and Secretions

5 1 LIVER

Cholinesterases occur in most tissues as evidenced by enzyme activity in homogenates or extracts, or by histochemical techniques The molecular properties of the enzymes have however been studied in only a few cases

The liver is generally assumed to be the site of production of serum cholinesterase (*Faber* 1941 1943 a, b *Sawyer* and *Everett* 1947, *Gerebtzoff* 1959) This assumption is based on indirect evidence in that the level of serum cholinesterase is low in certain liver diseases (*Antopol Schifrin* and *Tuchman* 1937) and after experimental liver damage (*Brauer* and *Root* 1946) The correlation between liver and serum cholinesterase is also indicated by the absence of cholinesterase activity in both liver biopsy specimens and serum of a patient which probably was of the silent" gene type see section 4 2 1 (*Doenicke et al* 1963 *Gürtner Kreutzberg* and *Doenicke* 1963) The correlation between the level of cholinesterase and of albumin in human serum (*Faber* 1941 1943 b) has also been taken as evidence for the hepatic origin of the enzyme On the other hand it has been shown in dogs that extirpation of the liver does not affect the level of serum cholinesterase (*Ginsberg Kohn* and *Necheles* 1937) the activity of the esterase was, however determined by a biological method which may have been unprecise The identity of liver and serum cholinesterases with respect to enzymatic properties (*Sawyer* 1945 *Sawyer* and *Everett* 1947 *Augustinsson* 1948 *Svensmark* 1963 b c) is consistent with the assumption of a hepatic origin of the serum enzyme and studies of the molecular properties of liver cholinesterases have not yielded evidence against this view

Starch-gel electrophoresis at pH 7 2 of extracts of human liver shows a broad band of cholinesterase activity with migration rates identical with and lower than the migration rate of human serum cholinesterase (*Ecobichon* and *Kalow* 1961 1962 1963 b) indicating the presence of more than one fraction of the enzyme In chromatography on DEAE-cellulose of human liver

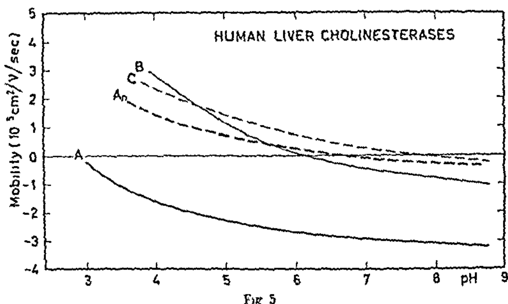


Fig 5

Electrophoretic mobilities as a function of pH at 1 C of three cholinesterase fractions (A B C) from human liver

The mobility was determined in paper electrophoresis in sodium acetate (pH 3–5.5) and sodium acetate + barbital buffer (pH 6–9), ionic strength 0.08–0.10 (*Svensmark* 1963 c) The mobilities of native fraction A were identical with those of native human serum ChE The mobilities of neuraminidase treated fraction A (A_n) were identical with those of neuraminidase treated serum ChE The mobilities of fraction B and C were not affected by treatment with neuraminidase Fraction B was of lower molecular weight than fraction C

TABLE II

Molecular properties of three fractions (A B C) of human liver cholinesterase and of native and sialic acid free human serum cholinesterasse (*Svensmark* 1963 c)

Enzym	Molecular weight	Electrophoretic mobility at 1 C pH 8.6 (cm²/V/sec)	Isoelectric point (pH)	Increase in mobility after neuraminidase
Liver cholinesterase				
fraction A	≳200 000	-3.1×10^{-5}	2.9	+
fraction B	<200 000	-1.0×10^{-5}	6	−
fraction C	≳200 000	-0.2×10^{-5}	8	−
Serum cholinesterase				
native	≳200 000	-3.1×10^{-5}	2.9	+
sialic acid free	≳200 000	-0.4×10^{-5}	6.5	−

extracts the cholinesterases were separated into two main fractions, one of which could be further separated on Sephadex G 200 into two fractions of different molecular weight (*Svensmark* 1963 b, c) Further studies on the properties of the three fractions (A B C) indicated that they are different molecular species (Table II Fig 5) On the other hand their enzymatic properties were identical with respect to (1) catalytic activity against butyryl choline acetylcholine and benzoylcholine, (2) inhibition by neostigmine succinylcholine and ASTRA 1397 and (3) activation by ammonium chloride. It is therefore possible that the three fractions represent different molecular forms of the same enzyme. Table II and Fig 5 show that fraction A is identical in all respects with native human serum cholinesterase Fractions B and C are different both from native and from sialic acid free human serum cholinesterase fraction B is remarkable in being of lower molecular weight than the other fractions of liver cholinesterase The findings are consistent with the idea of a hepatic origin of serum cholinesterase fraction A representing the final product, and the other two fractions representing precursors. The possibility remains, that fractions B and C are degradation products of fraction A, but incubation of liver homogenates for up to 10 days at 25° C did not lead to an increase of fractions B and C at the expence of fraction A.

Starch gel electrophoresis of liver extracts from man and other vertebrates showed several fractions of esterase, though these were not investigated thoroughly with respect to enzymatic properties. Most of the fractions may be aryl or carboxylesterases (*Hunter* and *Markert* 1957, *Allen Eräkö* and *Hunter* 1958 *Hunter* and *Burstone* 1958 1960 *Markert* and *Hunter* 1959 *Paul* and *Fottrell* 1961 a, b)

5 2 BRAIN AND SPINAL FLUID

Brain tissue contains cholinesterase as well as acetylcholinesterase (*Ord* and *Thompson* 1952) Cholinesterase occurs mainly in the cytoplasmic fraction (*Holmstedt* and *Toschi* 1959) and has been assumed to be associated with glial tissue (*Cavanagh Thompson* and *Webster* 1954) Electrophoresis of human brain extracts (from mixed grey and white matter) at pH 8 6 on paper or on cellulose columns demonstrated two cholinesterases (*Svensmark* 1960 1961 c) One fraction migrated like human serum cholinesterase and another at a lower rate together with γ-globulins. One fraction of acetyl cholinesterase migrated at an intermediate rate. The two cholinesterase fractions could be separated by chromatography on DEAE-cellulose The fractions were identical with respect to splitting of butyrylcholine butyrylthio-

choline and benzoylcholine, and to inhibition by neostigmine and ASTRA 1397. Acetyl-β-methylcholine was not split by any of the fractions. It might be suspected that the slowly migrating fraction is sialic acid free cholinesterase which might be formed in the presence of neuraminidase (see section 3.1.3.). Enzymic release of sialic acid from brain homogenates (man, ox, and guinea pig) at pH 3.5–4.0 indicates the presence of neuraminidase in brain (*Morgan* and *Laurell* 1963).

Starch gel electrophoresis of extracts of brain tissue from man and from various vertebrates has demonstrated the presence of several esterase fractions. In only one of these studies were the fractions investigated with respect to enzymatic properties, and it was found that three enzymatically identical acetylcholinesterases occur in human nucleus caudatus (*Bernsohn, Barron* and *Hedrick* 1963). In one study specific staining procedures were applied (*Lagnado* 1962), whereas in other studies the type of esterase was not determined (*Barron, Bernsohn* and *Hess* 1961, 1962, *Bernsohn, Barron* and *Hess* 1962, *Eränkö, Kokko* and *Söderholm* 1962 a, b, *Gerhardt, Clausen* and *Andersen* 1963, *Gerhardt et al.* 1963 a, b).

Spinal fluid in humans contains both acetylcholinesterase and cholinesterase (*Glasson* and *Mutrux* 1946, *Ress* and *Hemphill* 1948). The enzymes were separated by electrophoresis on paper at pH 8.6 and by chromatography on DEAE cellulose (*Svensmark* 1958, 1960, 1961 c). One fraction of cholinesterase migrated as does human serum cholinesterase, i.e. between α- and β-globulin, and the other fraction as does γ-globulin. The acetylcholinesterase migrated as does the τ-globulin of spinal fluid, or slightly slower than β-globulin. The two cholinesterases catalyzed the splitting of butyrylcholine and were inhibited by neostigmine and ASTRA 1397. Acetyl-β-methylcholine was not split. Both fractions are thus enzymatically similar to, if not identical with, serum cholinesterase in agreement with the assumption that spinal fluid proteins are derived from serum. The occurrence of a slowly migrating cholinesterase—which does not normally occur in serum—might be explained by the presence of neuraminidase in spinal fluid. This was in fact indicated by the finding of a change in mobility of serum cholinesterase when incubated with concentrated pools of spinal fluid from neurological patients (*Svensmark* 1961 c). Similarly the occurrence of free sialic acids in spinal fluid (*Uzman* and *Rumley* 1956) suggests that neuraminidase normally occurs in spinal fluid. I was, however, unable to find neuraminidase in three concentrated pools of spinal fluid from neurosurgical patients, and it is possible that neuraminidase occurs only in certain pathological spinal fluids. In fact, neuraminidase is produced by a number of microorganisms, e.g. *vibrio cholerae*, influenza virus, certain *pneumococci*, etc.

5 3 OTHER TISSUES AND SECRETIONS

The electrophoretic properties of cholinesterases in tissues other than liver and brain have been studied only in starch gel Extracts of mammalian retina contained 2–3 bands of acetylcholinesterase (man, cat cow, dog guinea pig, horse, pig, rabbit rat and sheep) and 1–3 bands of cholin esterase (man dog horse, pig and sheep) (*Estla* 1963) A fraction of cholin esterase in human kidney was suggested to be due to serum trapped in the tissue (*Ecobichon* and *Kalow* 1964) In a number of studies on different tissues the esterases were not characterized with respect to substrate specificity or other enzymatic properties (*Hunter* and *Markert* 1957, *Allen Eränkö* and *Hunter* 1958, *Markert* and *Hunter* 1959, *Allen* and *Hunter* 1960 *Paul* and *Fottrell* 1961 a b)

Cholinesterase has been found in milk of man dog cow, and swine (*McCance et al* 1949 *Hines* and *McCance* 1953 *Forster Montgomery* and *Montoure* 1961, *Heyndrickx* 1963) Electrophoresis on cellulose columns at pH 8 4 showed that the cholinesterase in sow s milk migrated much slower than the corresponding enzyme from plasma Sow s colostrum contains two large fractions of cholinesterase one migrating as does the plasma enzyme and another as does the milk enzyme in addition a smaller fraction migrated at an intermediate rate (*Augustinsson* 1958 b *Augustinsson* and *Olson* 1959) It was suggested by the authors that the slow migration of the enzyme in milk was due to an association with microsomes However, when mixed with plasma the milk enzyme migrated faster indicating an association with other proteins (*Augustinsson* and *Olson* 1959) The slowly migrating fraction may represent a sialic acid free enzyme (*Svensmark* 1961 c)

Pancreatic juice of dogs contains high cholinesterase activity (*Ginsberg Kohn* and *Necheles* 1937) *S afran S afran* and *Oleksy* (1959) reported separation of the enzyme into two electrophoretic fractions a finding not confirmed in their later studies (*S afran* and *S afran* 1963)

5 4 MULTIPLE FORMS OF TISSUE CHOLINESTERASE

Starch gel electrophoresis of extracts from human and animal tissues invariably has shown a multitude of different esterases (see sections 5 1, 5 2 and 5 3) Unfortunately these fractions have not been defined in detail and it is not possible with certainty to distinguish between cholinesterases and other esterases Furthermore the results of some of these studies are difficult to evaluate and to compare due to incomplete information as to pH or ionic composition of the buffer, site of application of the sample direction

of current, electric field (*Hunter* and *Markert* 1957, *Markert* and *Hunter* 1959, *Bernsohn Barron* and *Hess* 1961, *Paul* and *Fottrell* 1961 a b *Eränkö Kokko* and *Söderholm* 1962 *Gerhardt et al* 1963 b), or due to the interpretations of the effect of inhibitory agents (*Gerhardt Clausen* and *Andersen* 1963, *Gerhardt et al* 1963 b)

Molecularly different forms of enzymatically identical cholinesterases have been observed in human brain (and spinal fluid) and in human liver (*Svensmark* 1958, 1960 1961 c 1963 b, c) It is uncertain whether these forms represent native proteins precursors or degradation products, or all (see section 5 1 and 5 2)

With respect to the genetically controlled atypical human cholinesterase an important finding was published recently by *Liddell Newman* and *Brown* (1963) In liver, kidney, brain, ileum and skin from a homozygote for the atypical gene and from a heterozygote the type of cholinesterase was investigated and was found to be atypical and heterozygous respectively This finding indicates that the same molecular species is responsible for the cholinesterase in the different tissues and that its synthesis is controlled by the same genes or that the synthesis takes place at one locus

6 Perspectives

Study of the molecular properties of the cholinesterases aims at an understanding of the relation between structure and function of the enzyme Cholinesterases are tempting objects of such studies because a great variety of enzymes with slightly different catalytic properties occurs in various species and individuals. The study of acetylcholinesterases seems particularly rewarding since it may imply not only the catalytic function of the enzyme but also structural changes of the protein during catalysis There is evidence that acetylcholinesterases are integral parts of certain cell membranes, and binding and release of substrate could be associated with changes in the permeability of the excitable membranes in axons synapses and effector-organs

It is obvious from this review that knowledge of the molecular properties of cholinesterases is still incomplete The most promising results have been obtained in studies of the active site of cholinester hydrolases, which hitherto have shown that histidine and serine are essential components of the esteratic site The possibilities of such studies have been far from exhausted and new knowledge may be anticipated from investigations of the effect of chemical modifications of cholinesterases This line of work has the advantage that pure preparations of the enzyme are not needed

On the other hand a complete structural analysis requires pure preparations of the enzymes which are not yet available Therefore the purification of the enzymes is an indispensable goal of future studies. There is reason to assume that the purification can be accomplished by conventional procedures though the small concentrations of the enzymes in natural sources presents a serious complication It may be worth while to investigate whether induction of cholinesterase formation in bacterical cultures may provide a richer source

The fact that the cholinesterase molecule has a molecular weight of more than 200 000 makes it an inconvenient object of structural analysis, and motivates a search for active sub-units The removal of catalytically inert sialic acid residues from the molecule may be a first step in this direction and attempts should be made to remove other sugar components as well

since a naked protein molecule might be more easily subdivided The existence of active sub-units is indicated by the findings of cholinesterase fractions of low molecular weight in serum (*Harris* and *Robson* 1963 a) and in liver (*Svensmark* 1963 c)

Summary

The molecular properties of cholinesterases (acylcholine acyl hydrolase EC 3 1 1 8 formerly pseudocholinesterase non specific cholinesterase) have not been studied extensively mainly due to the lack of pure preparations of the enzymes Valuable information has however been gained indirectly using their catalytic activity to identify the enzymes in electrophoretic and chromatographic studies Further information on the components of the molecules has been obtained from the effect of interactions with more or less specific agents on the physical and enzymatic properties of the enzyme

Serum cholinesterases from man and horse are highly soluble in water precipitable by 2–3 M ammonium sulphate and soluble in the presence of zinc ions The molecular weight of human serum cholinesterase has been estimated to be about 300 000 For horse serum cholinesterase the molecular weight per active site has been estimated to be less than 84,000 The electrophoretic mobility of cholinesterases has been determined by paper electro phoresis (Fig 2–4) The isoelectric point for human serum cholinesterase was at pH 2 9 and for equine serum cholinesterase at pH 3 1 in acetate and phosphate buffers (ionic strength 0 08–0 1) The mobility at pH 8 6 (barbital buffer) was $-3\ 1\ \ 10^{-5}$ cm /V/sec (man) and $-2\ 7\ \ 10^{5}$ cm /V/sec (horse) *i e* between the mobilities of α and β globulin The course of the mobility pH curves somewhat resemble that of human orosomucoid with a steep slope below pH 3 5–4 and a rather flat course above pH 4 The curve may indicate the presence of a large number of sialic acid residues and relatively few carboxylic amino-, imidazole and tyrosine hydroxyl groups

The electrophoretic mobilities of serum cholinesterases in other species are not known but *determinations of the relative migration of serum cholin* esterases of some vertebrates indicate that the properties of the enzymes in lower vertebrates differ from those of human serum cholinesterase

There is nothing to indicate catalytically active prosthetic groups in the structure of serum cholinesterases Though the presence or absence of metals modifies the activity of the enzyme, metals are not an absolute requirement for activity Treatment of human serum cholinesterase with neuraminidase

did not affect the catalytic properties of the enzyme, but a considerable increase in electrophoretic mobility at pH 3–9 indicated that several sialic acid residues were removed from the enzyme The polypeptide portion of the molecule was hardly affected by the treatment since the slope of the mobility curve above pH 4 was unchanged, the change observed below pH 4 is due to the removal of sialic acid residues rather than carboxyl groups Similar results were obtained with horse serum cholinesterase (Fig 4)

Degradation of horse serum cholinesterase, labelled at the active site with radioactive diisopropylfluorophosphate, yields peptides with the amino acid sequence phenylalanin—glycine—glutamic acid—phosphorylated serine—alanine—glycine followed by two alanine and one serine residues in unidentified sequence Indirect evidence points to histidine as a part of the active site of the enzyme, there is no evidence of sulphydryl groups

Electrophoretic chromatographic and kinetic studies indicate that human and equine sera contain more than one cholinesterase with only slightly different properties A genetically controlled cholinesterase in human serum—atypical human serum cholinesterase—has been investigated more thoroughly Its substrate and inhibitor specificity differs from that of the normal enzyme whereas its electrophoretic mobility before and after treatment with neuraminidase is identical with that of the normal enzymes

Tissue cholinesterases are partly extractable In human *brain* (and *cerebrospinal fluid*) two cholinesterases can be separated electrophoretically and chromatographically The two fractions are identical with respect to enzymatic properties but differ in electrophoretic mobility One migrates as serum cholinesterase and the other as *γ* globulin, *i e* as sialic acid free serum cholinesterase Whether this fraction occurs as a result of the action of neuraminidase cannot as yet be decided In cerebrospinal fluid neuraminidase was found in some but not in all pools investigated

In human *liver* three enzymatically identical cholinesterases could be separated by electrophoresis and chromatography One is identical with serum cholinesterase with respect to content of sialic acid and electrophoretic mobility The two others differ with respect to mobility and they are not sialoproteins one had a lower molecular weight than the other fractions The two latter fractions may represent precursors of human serum cholinesterase, and the findings are consistent with the assumption that serum cholinesterase is produced in the liver

Cholinesterases in *other tissues* have not been thoroughly investigated with respect to molecular properties A cholinesterase in sow's colostrum exhibits electrophoretic properties which indicate that it is a sialic acid free cholinesterase

Our knowledge of the molecular properties of cholinesterases is thus limited and there is a long way to the final goal of investigations of this kind, the detailed analysis of the molecule and the chemical synthesis of an active enzyme

Dansk sammenfatning

(Danish Summary)

Der findes to typer af cholinester hydrolaser 1) *Acetylcholinesterase* (acetylcholin acetyl hydrolase, EC 3.1.1.7, tidligere benævnt specifik cholinesterase) og 2) *cholinesterase* (acylcholin acyl hydrolase, EC 3.1.1.8, tidligere benævnt bl. a. pseudo-cholinesterase og ikke specifik cholinesterase).

Acetylcholinesteraser findes i nervevæv og i somatiske og autonome effektorer, f. eks. motoriske endeplader, hvor de katalyserer spaltningen af acetyl-cholin som frigøres ved overføringen af nerveimpulser i visse synapser samt til muskler og visse autonome effektorer. Lignende enzymer findes hos elektriske fisk, hvor de synes at være af betydning for produktionen af elektrisk energi i de elektriske organer. Endvidere findes acetylcholinesteraser ved overgangen mellem muskelfibre og sener, i visse slangegifte, i røde blodlegemer og thrombocyter, hvor deres funktion ikke er kendt.

Cholinesteraser findes i ganglier, plasma, glia, lever, tarm, muskel, lunge samt i pankreas og andre kirtler. Deres fysiologiske funktion er ukendt. Man kan hæmme cholinesterase fuldstændigt uden at der kan iagttages farmakologiske virkninger på organismen. Dog er det muligt, at cholinesteraser spiller en rolle for tarmbevægelser. Der foreligger mange spekulationer over enzymets fysiologiske rolle. Man har formodet, at det fungerede ved at nedbryde acetylcholin eller andre cholinestere, som enten undslap dannelsesstedet i nervesystemet eller blev dannet uden for dette. Man har endvidere gættet på, at enzymet var af betydning for cholinstofskiftet, for myeliniseringen, for transporten af næringsstoffer i leveren eller for opbygningen af acetylcholinesteraser.

Cholinesteraser findes hos forskellige arter og individer i et stort antal varianter med lidt afvigende katalytiske egenskaber og udgør derfor et alsidigt materiale for sammenlignende undersøgelser mellem katalytiske og strukturelle egenskaber. For acetylcholinesterasernes vedkommende ville sådanne undersøgelser måske vise sig at have endnu videre perspektiver, idet den katalytiske

funktion af enzymet kan tænkes at være knyttet til strukturelle ændringer i proteinet eventuelt sådanne som har sammenhæng med permeabiliteten i de exitable membraner i axoner, synapser og effektor organer

Cholinesterasernes katalytiske egenskaber er særdeles vel undersøgte, mens deres molekylære egenskaber kun er lidt kendte Formalet med denne over sigt har været at samle de foreliggende data vedrørende cholinesterasernes molekylære egenskaber Da acetylcholinesteraserne ikke forekommer naturligt i oploselig form, foreligger der kun få og usikre undersogelser over deres molekylære egenskaber og de er derfor ikke medtaget i oversigten

Cholinesterase er endnu ikke præpareret i ren tilstand hvorfor man ikke har kunnet foretage direkte strukturanalyser pa enzymet Man kan dog ad indirekte vej opnå oplysninger om enzymets molekylære egenskaber idet man ved hjælp af enzymets katalytiske aktivitet kan lokalisere proteinet i elektroforetiske og kromatografiske undersogelser Endvidere har man udfra virkningen af forskellige stoffer pa enzymet kunnet drage visse slutninger med hensyn til molekylets opbygning

Serum cholinesterase fra menneske og hest er letoploselige i vand ogsa i nærværelse af zinkioner Enzymerne fældes af 2–3 M ammoniumsulfat Molekylvægten for human serumcholinesterase er ca 300 000, og for heste-cholinesterase over 200 000 84 000 g af en 14 000 gange renset præparation af heste serumcholinesterase forbinder sig med 1 mol diisopropylfluorophosphat (DFP) og da 1 mol DFP forbinder sig med 1 mol serin i enzymets »aktive omrade« (»active site«) må molekylvægten per aktivt område være mindst 84 000 (*Jans.* og *Cohen* 1961) Enzymet ma altsa antages at indeholde 2–4 aktive omrader pr molekyle

Serumcholinesterasernes elektroforetiske mobilitet er bestemt ved hjælp af papirelektroforese (*Svensmark* og *Kristensen* 1963) For human serumcholinesterase var det isoelektriske punkt ved pH 2 9, og for det tilsvarende enzym hos hesten ved pH 3 1 (i acetat og phosphatbuffer, ionstyrke 0 08–0 1) Mobiliteten ved pH 8 6 (diemalbuffer) var $-3\ 1 \quad 10^{5}$ cm²/V/sec (menneske) og $-2\ 7 \quad 10^{-5}$ cm²/V/sec (hest) d v s imellem mobiliteterne for α og β globulin

Mobilitets pH kurverne ligner den tilsvarende kurve for human orosomucoid (se Fig 2 side 17) idet hældningen er stor ved pH værdier under 3 5–4 og lille ved pH værdier over 4 Orosomucoids elektroforetiske egenskaber er i det væsentlige bestemt af dette stofs store antal af sialsyre rester og det relativt ringe antal carboxyl, amino imidazol og tyrosinrester, og det er rimeligt at antage en lignende sammensætning for serumcholinesterase

De elektroforetiske egenskaber af serumcholinesteraser fra andre arter er ikke nojere kendte men bestemmelser af relative vandringshastigheder har

Dansk sammenfatning

(Danish Summary)

Der findes to typer af cholinester hydrolaser 1) *Acetylch*
tylcholin acetyl hydrolase, EC 3 1 1 7, tidligere benævnt spe
rase) og 2) *cholinesterase* (acylcholin acyl hydrolase, EC 3
benævnt bl a pseudo-cholinesterase og ikke specifik cholineste

Acetylcholinesteraser findes i nervevæv og i somatiske og au
torer f eks motoriske endeplader, hvor de katalyserer spaltnin
cholin som frigøres ved overføringen af nerveimpulser i visse syn
muskler og visse autonome effektorer Lignende enzymer findes h
fisk hvor de synes at være af betydning for produktionen af el
i de elektriske organer Endvidere findes acetylcholinesteraser ved
mellem muskelfibre og sener i visse slangegifte, i røde blodlegeme
bocyter hvor deres funktion ikke er kendt

Cholinesteraser findes i ganglier plasma glia lever, tarm m
samt i pankreas og andre kirtler Deres fysiologiske funktion er ul
kan hæmme cholinesterase fuldstændigt uden at der kan iagtt
kologiske virkninger på organismen Dog er det muligt at choli
spiller en rolle for tarmbevægelser Der foreligger mange spekulatio
enzymets fysiologiske rolle Man har formodet at det fungerede ved
bryde acetylcholin eller andre cholinestere som enten undslap dann
i nervesystemet eller blev dannet uden for dette Man har endvidere g
at enzymet var af betydning for cholinstofskiftet, for myeliniserin
transporten af næringsstoffer i leveren eller for opbygningen af acety
esteraser

Cholinesteraser findes hos forskellige arter og individer i et stort
varianter med lidt afvigende katalytiske egenskaber og udgør derfor et
materiale for sammenlignende undersøgelser mellem katalytiske og struk
egenskaber For acetylcholinesterasernes vedkommende ville sådanne u
søgelser måske vise sig at have endnu videre perspektiver, idet den katal

tioner har endvidere en lavere molekylvægt end serumcholinesterase Det foreslås, at de to sidstnævnte fraktioner repræsenterer forstadier til serum cholinesterase, som formentlig dannes i leveren

*

Vor viden om cholinesterasernes molekylære egenskaber er således meget begrænset Der er lang vej til det endelige mål for sådanne undersøgelser en strukturbestemmelse og en kemisk syntese af et aktivt enzym Man må i første række søge at fremstille enzymet i ren tilstand rensningen vil antageligt kunne gennemføres med konventionelle metoder men de lave koncentra tioner, i hvilke enzymerne forekommer vil være en alvorlig komplikation Da molekylvægten for enzymet er mere end 200 000 vil en detailleret struk turanalyse – selv med det rene enzym til rådighed – være så kompliceret at man bør søge at nedbryde det til mindre aktive enheder Måske vil fjer nelsen af sialsyre vise sig at være et skridt i denne retning Molekylet må antages at indeholde andre sukkerstoffer som eventuelt også vil kunne fjer nes hvorefter det »nøgne« protein vil være tilgængeligt for en forsigtig ned brydning Fundet af en lavmolekylær cholinesterasefraktion i human serum (*Harris* og *Robson* 1963 a) og i human lever (*Stensmark* 1963 c) gør det sandsynligt at der findes aktive under enheder

Appendix

Reviews on cholinester hydrolases[1])

The main subjects of the reviews are indicated by the following abbreviations

physiol.	physiological function
pharm.	pharmacology and toxicology
distr.	distribution in organs and species
hist.	histology and cytological distribution
kin.	kinetics
inh.	inhibitors
mol.	molecular properties
site	active site
hydr.	mechanism of hydrolysis
meth.	methods

References	ACbE	CbE	Related enzymes	Subjects
Aldridge 1956	+	+		inh. site
Ammon 1943	+	+		inh.
Ar[illegible] 1958	+	+	+	physiol. distr. hist. inh. meth.
Augustinsson 1948	+	+	+	physiol. distr. kin. inh. meth.
Augustinsson (1950)	+	+	+	physiol. distr. kin. inh. meth.
Augustinsson 1954	+	+		pharm. kin. inh.
Augustinsson 195[illegible]	+	+		meth.
Augustinsson 1960a		+	+	distr. kin. inh. mol. site hydr. meth.
Augustinsson 1961		+	+	distr. mol.
Augustinsson 1963	+	+	+	distr. inh. mol. site meth.
[illegible] and Stein 195[illegible]	+	+	+	site hydr.
Bergmann 1955	+	+		kin. inh. site. hydr.
Bergmann 195[illegible]	+	+		kin. inh. site. hydr.
B[illegible] and Mac[illegible] 1955	+			physiol. distr.
Chadwick 1963	+			physiol. pharm. inh.
Cohen and Oosterbaan 1963	+	+	+	kin. inh. site. hydr.
Cohen et al. 1[illegible]	+	+	+	site hydr.
Cohen et al. 1[illegible]	+	+	+	site hydr.
C[illegible] 1963	+			physiol. pharm. inh.
Da[illegible] 1[illegible]	+	+		pharm. inh.

[1]) For reviews on the active site of hydrolases see section 3.5.1.1, and on cholinesterase variants section 4.2.1.

References	AChE	ChE	Related enzymes	Subjects
Davies (1963)	+			physiol pharm inh
Davies and Green (1958)	+	+	+	kin inh site hydr
Du Bois (1963)	+	+		pharm inh
Feldberg (1945)	+	+		physiol distr
Gereb off (1959)	+	+		physiol distr hist meth
Giacobini (1959)	+	+		distr hist.
Hardegg (1958)	+	+		kin
Heath (1961)	+	+	+	inh pharm
Hebb (1957)	+			physiol distr
Hebb and Krnjevic (1963)	+			physiol distr
Hobbiger (1963)	+			pharm inh
Hofstee (1960)			+	distr kin inh
Holmstedt (1951)	+	+		physiol pharm. inh
Holmstedt (1959)	+	+		physiol pharm inh
Holmstedt (1963)	+	+		inh
Jans et al (1963)	+	+	+	site hydr
Karczmar (1963a)	+			physiol distr
Karczmar (1963b)	+			physiol pharm inh
Koelle (1963b)	+	+		physiol distr hist meth
Koelle and Gilman (1949)	+	+		physiol pharm distr inh
Koshland (1960)	+	+	+	site. hydr
Lévy (1947)	+	+		physiol. distr inh meth
Long (1963)	+	+		inh
Machne and Unna (1963)	+			physiol pharm inh
Myers (1960)	+	+	+	hydr
Nachmansohn (1945)	+			physiol distr
Nachmansohn (1955)	+			physiol distr inh site hydr
Nachmansohn (1959)	+			physiol inh site hydr
Nachmansohn (1962)	+			physiol inh site hydr
Nachmansohn (1963)	+			physiol pharm. inh
Nachmansohn and Wilson (1951)	+			physiol kin inh site hydr
Nachmansohn and Wilson (1955)	+			physiol distr inh site hydr
O Brien (1960)	+	+	+	physiol pharm inh. site
Schaefer (1947)	+	+		kin inh
Thompson (1954)	+	+		physiol distr kin. inh
Vincent (1955a b)	+	+		physiol pharm distr kin inh
Werner and Kuperman (1963)	+			physiol pharm inh
Whittaker (1951)	+	+		physiol distr kin inh site hydr
Wilson (1954)	+			physiol kin inh site hydr
Wilson (1955)	+			physiol kin inh site hydr
Wilson (1960)	+			physiol kin inh site hydr
Zaimis (1963)	+			physiol pharm inh
Zeller (1948)	+			physiol distr inh

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Accelerated Transfer of Oxygen through Solutions of Heme Pigments

BY

EDVARD A HEMMINGSEN

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FROM THE PHYSIOLOGICAL RESEARCH LABORATORY
UNIVERSITY OF CALIFORNIA SAN DIEGO
CALIFORNIA U S A

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OSLO 1965

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E H



Introduction

It has been shown that when a solution of hemoglobin held in a membrane is exposed to an oxygen gradient the rate at which oxygen passes through the solution may under certain circumstances be accelerated above the rate of its movement through water Prior to this demonstration theoretical considerations led ROUGHTON and his associates (ROUGHTON 1932 1959 KLUG, KREUZER and ROUGHTON 1956a b) to suggest that diffusion of oxyhemoglobin might speed up the diffusion of oxygen in solutions of the pigment In a comprehensive series of investigations on the kinetics of oxygen penetration into hemoglobin solutions (LASZT 1945 MULLER 1948 KREUZER 1953 KLUG *et al* 1956a b and others) the gross parameters were laid down empirically as well as theoretically but the enhancement effect, which is the subject of the present treatise was not perceptible or was only dimly so in the empirical data Their photometric technique, operating through layers of hemoglobin solution less than 0 2 mm thick was admirably suited for their purpose but did not have sufficient resolving power for this particular problem Increased passage of oxygen through hemoglobin containing agar has been reported by WITTENBERG (1959)

Only when approached with a steady state technique did the effect stand out in full clarity (SCHOLANDER 1960 HEMMINGSEN and SCHOLANDER 1960) The hemoglobin solution was contained in a thin porous membrane which was subjected to various constant pressure differentials of air, oxygen or nitrogen and the transfer rates of the gases through the membrane were measured volumetrically This method had important advantages it was not hampered by the large oxygen capacity of the system and both oxygen and the control nitrogen rates were determined directly and simultaneously

The results revealed that a great increase in the rate of oxygen transfer through the hemoglobin solution was manifested when one side of the system was maintained at zero oxygen pressure The oxygen rate was up to 8 times the rate through a methemoglobin solution while the nitrogen rates in the two solutions were the same The oxygen transfer enhancement was selectively impeded by increased viscosity, and was greatly reduced when gelatin was added to the solution This strongly suggested that the thermal motion of the pigment was responsible for the increased transfer Accelerated transfer has likewise been demonstrated for myoglobin solution (SCHOLANDER 1960 HEMMINGSEN 1963) and for carbon monoxide through hemoglobin solution (MOCHIZUKI and FORSTER 1962)

The empirical data have been discussed by several authors in terms of conventional diffusion principles ascribing the effect to diffusion of oxyhemoglobin (COLLINS 1961a, b WANG 1961, 1963, FATT and LA FORCE 1961 MOCHIZUKI and FORSTER 1962) SCHOLANDER (1960) suspected that a significant part of the membrane might be saturated when the enhancement prevailed and considered that the transfer may take place through chains of colliding hemoglobin molecules A similar concept was formalized by FYNS (1964) who suggested that the oxygen may be transferred via hemoglobin by a mechanism derived from the theory of heat transfer These concepts will be discussed later in connection with further empirical data

The transfer of oxygen through hemoglobin solution represents carrier transport in its simplest form The appeal of this particular system is that the carrier (hemoglobin) and its cargo (oxygen) are well known indeed and that it lends itself to studies under well controlled conditions It is a unique advantage that the substance being transferred is a gas and that it has an isotope which is suitable for tracer experiments

The scope of the present investigation is to widen the empirical knowledge of this system by new experimental data, and from this information to gain further insight into the molecular mechanism involved The emphasis has been put on the transfer by hemoglobin but data from other pigments primarily myoglobin, will also be presented In addition various supplementary experiments of secondary importance will be described The physiological implications will be considered briefly

Some of the methods and results have been reported in previous communications (HEMMINGSEN and SCHOLANDER 1960 HEMMINGSEN 1962 1963) and will here be introduced only as needed for a fuller discussion

Investigations on Hemoglobin Solutions

All diffusion experiments presented utilize the same basic system, namely porous membranes charged with solution The membranes were always suspended between two chambers in which various partial pressures of oxygen were obtained either by pure oxygen gas at the desired pressure or by oxygen nitrogen mixtures maintained at atmospheric pressure The gases introduced were water-saturated and in addition a piece of wet filter paper was kept in each chamber to minimize evaporation from the membrane The chambers provided with tubes for flushing with gas were of sufficient size to give near steady state conditions for a limited time period Temperature was controlled within $\pm 0\,2$ C by water jackets or by submersion in a water bath The various designs are illustrated in Figs 1, 3 and 11

PREPARATION OF HEMOGLOBIN SOLUTION AND MEMBRANES

Hemoglobin solutions were prepared from heparinized human blood The erythrocytes separated from plasma by centrifugation were repeatedly washed and suspended in 0 85 per cent saline solution equal in volume to the plasma removed Repeated freezing and thawing provided complete hemolysis The final solution was filtered and the oxygen capacity was determined by a syringe method (ROUGHTON and SCHOLANDER 1943) The capacity was adjusted with saline to 20–21 vol per cent Before use the *p*H was adjusted to desired values with sodium bicarbonate or lactic acid Methemoglobin solutions were obtained by adding a few crystals of potassium ferricyanide to 3–4 cm^3 of hemoglobin solution

The membranes used throughout this study were prepared by soaking Millipore filters in the solutions and wiping off the excess from both surfaces The Millipore filters grade HA with an average pore size of 0 45 μ and about 80 per cent void space (man-

ufacturer's information), prevent convection, and the capillarity cannot be broken by a gas pressure differential of 1 atmosphere The filters had a constant thickness of 0 015 cm The charged filters are hereafter called *membranes*

The problem arises as to whether the solution in the membranes can be treated as a free solution This becomes important in relation to physical and chemical parameters which have been determined for free solutions It is possible, for instance, that the thermal agitation of the hemoglobin molecules may be affected by adherence to the pore walls However, such an effect is not likely to be very large, and preliminary observations by a photometric method indicated that the diffusion of hemoglobin in the membranes was substantially the same as in free solution

OXYGENATION OF HEMOGLOBIN IN THE MEMBRANES

The dissociation curve The oxygen dissociation curves were determined directly in the membrane by a Zeiss Pulfrich Stufen Photometer, provided with a special cuvette (Fig 1)

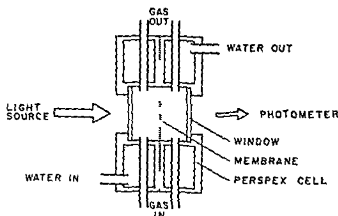


Fig 1 Cuvette used for determinations of dissociation curves and oxygenation gradients in membranes Exposed membrane area 1 cm²

The cuvette with membrane was placed in the light beam of the photometer and the same oxygen nitrogen mixture was admitted to both chambers by flushing The absorption in the membrane for each partial pressure of oxygen was read through a Wratten No 29F red filter and was compared with a reference gray filter This

red filter gives the greatest separation between reduced and oxygenated hemoglobin The method is readily adaptable to other types of spectrophotometers and requires very small amounts of liquid Stable readings were obtained 6 to 8 minutes after change of gas A slight drift possibly caused by evaporation was minimized by shielding the light beam between readings The ratio of oxygenated to reduced hemoglobin was calculated in the conventional way (ZIJLSTRA 1951) assuming that full reduction of the hemoglobin was obtained with nitrogen and full oxygenation with air

Fig 2 shows the dissociation curves for 2 of the stock solutions which were used in most of the diffusion experiments

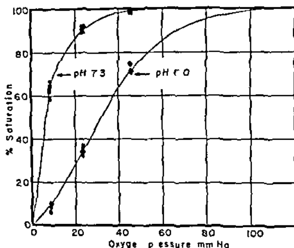


Fig 2 Oxygen dissociation curves of hemoglobin solutions suspended in membranes Temperature 25 C Each point represents duplicate measurements

The hemoglobin oxygenation gradient Transfer of oxygen through the membranes is closely linked with the oxygenation of hemoglobin A question of prime importance is whether (1) the oxygen tension or (2) the oxygenation of hemoglobin is linear when net flux enhancement prevails This information was obtained by a reflectance photometer which determined the oxyhemoglobin content at the membrane surfaces

The instrument was a custom built double beam type where the reflectance from the sample was compared to a constant reference surface The membrane was charged with hemoglobin solution of 20 or 10 vol per cent oxygen capacity and was suspended in a

cuvette similar to that shown in Fig. 1. Gas was slowly flushed through the two gas chambers in combinations of air/air, air/nitrogen, nitrogen/air, and nitrogen/nitrogen. The fraction of incident monochromatic light (5600 Å) reflected from one side of the membrane was determined for each of these combinations, and the corresponding oxyhemoglobin fraction was calculated. The transparency of the membrane causes light to be reflected not only from the surface, but from a layer below as well. Therefore the values obtained represent the amount of oxyhemoglobin in an undefined but constant layer of the membrane.

The concentrated solution gave 91 per cent oxygenation when air was maintained on the illuminated side and nitrogen on the dark side. With the gases reversed, the value was 28 per cent. The less concentrated solution which provided a deeper penetration for the light gave respectively 90 and 65 per cent oxyhemoglobin. The results show that the oxyhemoglobin is greatly displaced toward the nitrogen side, which is in the direction of a linear oxygen tension gradient rather than a linear oxyhemoglobin gradient.

Further investigations on the gradients have been performed in this laboratory (Enns 1964). The average content of oxyhemoglobin in membranes suspended between helium and various oxygen pressures was determined photometrically by means of light transmitted through the membranes with instrumentation similar to that used for the dissociation curve (Fig. 1).

It was found that when oxygen pressures exceeded the saturation point at the entrance surface the oxygenation was vastly higher than the 50 per cent which would be obtained with a *linear* oxygenation gradient. However, the observed values were substantially less than those expected from a linear tension gradient and complete equilibria. For instance, with an oxygen pressure of 150 mm Hg, the observed oxygenation was 79 per cent as compared to the expected 91 per cent.

NET TRANSFER OF OXYGEN WITHOUT BACK PRESSURES

The steady state transfer of oxygen through hemoglobin solutions, subjected to various oxygen pressures on one side and vacuum on the other, has previously been investigated by Scholander (1960). The apparatus employed is schematically illustrated in Fig. 3A. The

oxygen pressure on the lower side of the membrane was always zero i e moist vacuum, while air at various pressures ranging from $^1/_{12}$ to 1 atmosphere were maintained on the upper side The gas permeating the membrane accumulated in the large reservoir, and at intervals was collected measured volumetrically, and analyzed for oxygen and nitrogen [1]

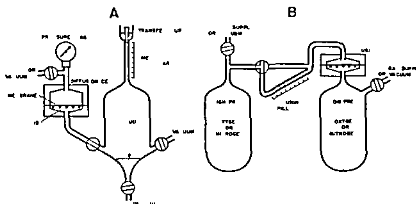


Fig 3 Instrumentation for volumetric net gas flux measurements
A For measurement of gas flux without back pressures
B For measurement of gas flux with back pressures

The results from one series of experiments are given in Fig 4 These data have been calculated to absolute units from the original determinations, previously presented only as relative values It will be seen that the net flux of oxygen through the control methemoglobin solution was strictly proportional to the pressure with the oxygen nitrogen ratio of the gas which permeated the solution remaining constant 0 54 This value is close to a ratio of 0 50 which would be expected for diffusion of air through water, and hence the oxygen proceeded through the solution by simple diffusion Relative to water however both the oxygen and nitrogen net fluxes were reduced (Table IV) The net flux of oxygen through hemoglobin solution was greatly increased compared with that through

[1] *Terminology and units* The terms introduced here will be used throughout this study *Flux* signifies the rate of transfer through unit area of the membrane in any direction *Net flux* is the resultant flux in a given direction The *flux enhancement* is the flux component in excess of that provided by simple diffusion When absolute values are of importance the flux is expressed in $cm^3 cm^{-2} sec^{-1}$ dry gas volume at 0° C and 760 mm Hg

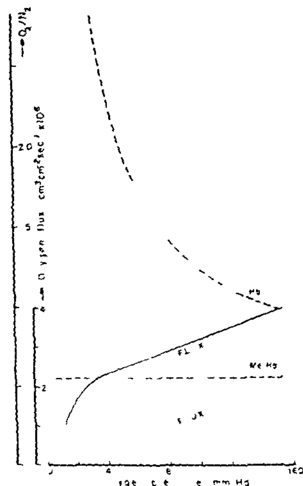


Fig 4 Steady state net flux of oxygen through hemoglobin and methemoglobin solutions without back pressures (Fig 3 A) Oxygen capacity 22 9 vol per cent temperature 24 C pH 7 3 Solid and dotted lines Flux curves Stippled lines oxygen nitrogen ratios (Recalculated from data by Scholander 1960)

methemoglobin solution A seven fold difference in absolute rates was manifested at low pressures and the oxygen nitrogen ratio directly reflected this difference Consequently only the oxygen was affected by the hemoglobin the nitrogen net flux was the same for the two solutions The net flux enhancement that is, the difference between the oxygen flux through hemoglobin and through methemoglobin solution was constant over a wide pressure range but decreased when the pressure dropped below the saturation point of the solution

NET TRANSFER OF OXYGEN WITH BACK PRESSURES

The preceding experiments demonstrated that the hemoglobin greatly increased the oxygen net flux when the pressure on one side of the membrane was zero What would happen to the transfer enhancement if the membranes were opposed by back pressures of oxygen with the pressure difference maintained as before? This was determined by volumetric measurements

The apparatus (Fig 3B) and experimental procedures have been described in detail elsewhere (HEMMINGSEN and SCHOLANDER 1960) Each of the two chambers in the diffusion cell was connected to a large gas reservoir the lower directly by a short tube the upper via a graduated capillary and a three way stopcock for resetting of the kerosene indicator drop With the membrane in place the apparatus was gently evacuated and the chambers were refilled with oxygen (or nitrogen) to desired pressures The permeation of gas through the membrane was determined by displacement of the indicator drop after constant rates were obtained Examples of these measurements are shown in Fig 5

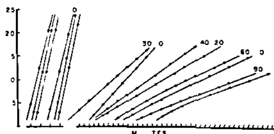


Fig 5 Volumetric measurements of gas flow (Fig 3 B) The observations are from two different membranes and illustrate the maximum spread in raw data Numbers above curves are pressures in the two chambers Flux is calculated from the slope 1 mm equals 2 66 mm³ membrane area is 7 8 cm²

The standardized net flux data are given in Fig 6 In the two series of measurements presented in the diagram the total pressures were varied but the pressure differential was kept constant at either 80 or 20 mm Hg The oxygen net flux was constant when the opposing oxygen pressure was 10 to 15 mm Hg or more and was about twice the nitrogen net flux The theoretical ratio which would be expected for simple diffusion of the gases through water is 1 9 and hence the oxygen was not specifically affected by the hemo

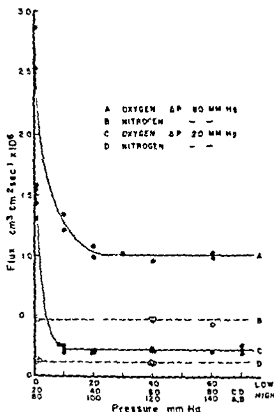


Fig 6 Net flux of oxygen and nitrogen through hemoglobin solution determinated by volumetric method (Fig 3 B) The abscissa represents pressures in the two chambers 6 membranes Oxygen capacity 21 vol per cent temperature 23 C

globin but proceeded through the solution by simple diffusion This is also borne out by the fact that the four fold difference in gradients between the two series was closely reflected in both the oxygen and the nitrogen net flux However when the opposing oxygen pressure is less than 10 mm Hg the oxygen net flux increased sharply while the nitrogen net flux remained unchanged Without back pressures the increase in oxygen net flux for the two series was respectively two and seven-fold The absolute enhancements were comparable to those obtained for similar gradients by the other volumetric method (Fig 4)

The absence of net enhancement when the solution was opposed by back pressures is also evident in Fig 7 In this series of experiments the oxygen (or nitrogen) pressure in the lower chamber was maintained constant at 20 mm Hg while the pressures in the

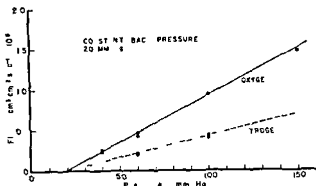


Fig 7 Net flux of oxygen and nitrogen through hemoglobin solution with constant back pressure of the respective gases 2 membranes

upper chamber were varied from 40 to 150 mm Hg The net flux of both gases was strictly proportional to the pressure gradient and the oxygen flux was twice that of nitrogen

The basic point emerging from these experiments is that the hemoglobin increases the oxygen net flux through the solution only when the oxygen pressure is so low that it leaves the pigment unsaturated on the exit side of the membrane The lowest opposing pressure which completely abolished the enhancement was 10 mm Hg or less at 20 mm Hg pressure gradient and about 15 mm Hg at 80 mm Hg pressure gradient At these back pressures, the hemoglobin would be almost fully oxygenated (Fig 2) The blocking was specific for oxygen for neither water vapor nor nitrogen impeded the enhancement

STUDIES WITH OXYGEN 18

This section will deal with the random mobility of molecular oxygen in hemoglobin solutions In these experiments oxygen 18 was employed as a tracer All isotope measurements were made by a mass spectrometer designed for these investigations (Fig 8) A short description of the instrument follows

THE MASS SPECTROMETER

The tracer experiments required determinations of oxygen 18/oxygen-16 ratios and of isotope concentrations directly on gas sam-

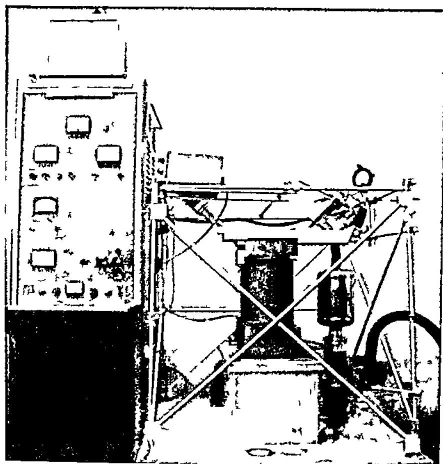


Fig 8 Photograph showing the complete mass spectrometer

ples maintained at atmospheric pressure It was desirable that the sample depletion by the instrument should be as small as possible The mass spectrometer was constructed with consideration to these requirements A sector-field type instrument offered the best practical advantages and by using a 90 magnetic field good mass resolution with adequate ion currents could be obtained at low sample concentrations The latter was important in order to avoid oxygen damage to the tungsten filament and to minimize the amount of gas removed from the sampled chamber A single ion beam collector system proved to be sufficiently accurate for the present determinations A schematic illustration of the instrument is shown in Fig 9 The ion source and the electronic components followed in principle the basic designs of Nier (1940, 1947)

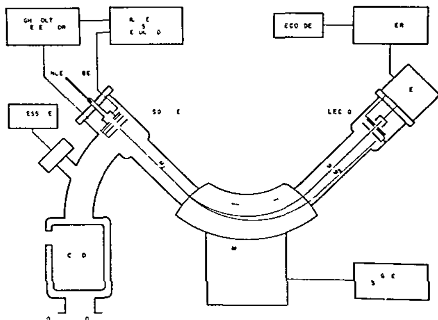


Fig 9 Diagram of mass spectrometer used in present work The radius of analyzer tube is 15 cm Magnet poles shown in correct proportions

In brief the instrument functions as follows The gas sample is admitted through a small leak to the ion source which is maintained at a pressure less than 10^{-6} mm Hg The ionized gas molecules are electrostatically accelerated through a series of slits into the magnetic field where they are deflected in circular paths the radii of which depend among other factors upon the mass and the electrical charge The intensity of the dispersed ion currents each containing ions of the same mass/charge ratio was measured separately by changing the accelerating voltage

The ion current was passed through a 10^{1} ohm resistor placed between the collector and the ground and the voltage developed across this resistor was measured with a 100 per cent negative feedback DC amplifier

The oxygen 18 enriched oxygen gave two main ion beams one containing $O^{16}O^{16+}$ (mass/charge $=$ m/e $=$ 32) the other $O^{18}O^{16+}$ (m/e $=$ 34) $O^{18}O^{18+}$ was negligible compared with the other two beams

The inlet system of the mass spectrometer was of the viscous flow type It consisted of a stainless steel tube about 2 meters long

and 0 25 mm inner diam , running directly from the sample chamber to the ion source The flow of gas was adjusted by inserting a 0 23 mm thick wire core into the tube for about 1 meter Time delay through the tube was about 15 seconds and the flow about 2 mm³ per minute

The relative isotope concentrations are given by the ion beam currents usually determined within ± 0 5 per cent It was established that there was no measurable fractionation of the two isotopes (Table I)

Table I

Determinations of oxygen isotopes in air

Air Sample	Atom Per Cent Oxygen 18	Accepted Value (Nier 1950)
I	0 204	
II	0 203	
III	0 204	0 204
IV	0 205	
V	0 205	

The relation between oxygen pressure and ion current (m/e = 32) was tested with various oxygen nitrogen mixtures and was found to be linear (Fig 10)

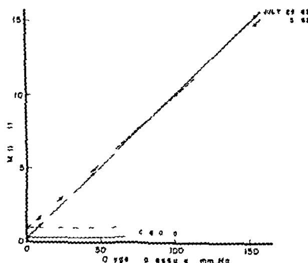


Fig 10 Examples of mass spectrometer calibration curves Each point represents several determinations within ± 0 5 per cent Pressure in sample chamber plotted against ion beam intensity (m/e = 32)

The mobility of oxygen in hemoglobin solution depends on its exchange with the pigment and it is therefore important to know how this exchange is affected by oxygenation Does a fully oxygenated hemoglobin molecule exchange its oxygen with water at all 4 sites or is the exchange more restricted? The well established concept of dynamic equilibrium (see ROUGHTON 1959 GIBSON 1959) implies the first to be the case but this aspect of the exchange has not been demonstrated empirically

In the following experiments hemoglobin solution was equilibrated with oxygen 18 enriched gas and the release of the isotope into a gas phase of either nitrogen or unlabeled oxygen was observed

Method and procedures The chamber with flushing tubes for gas is seen in Fig 11A It connected directly to the inlet tube of the mass spectrometer The solution in it (0 5 cm³) was continuously stirred and temperature controlled

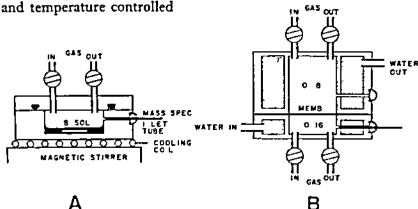


Fig 11 A Chamber used to determine exchange of oxygen 18 between hemoglobin and gas phase B Chamber used to determine flux of oxygen 18 through membranes Volume of upper chamber 64 7 cm³ lower chamber 12 8 cm³ Membrane area is 11.8 cm²

After repeated flushing with nitrogen the solutions were equilibrated with an oxygen nitrogen mixture maintained at atmospheric pressure with the partial pressure of oxygen being 46 mm Hg and the oxygen 18 concentration 1 66 atom per cent The gas above the solution was quickly switched to either (1) pure nitrogen or (2) a

mixture of the same composition as before, but with oxygen-18 in natural abundance (0 204 atom per cent) The release of oxygen-18 from the solution was recorded by the mass spectrometer

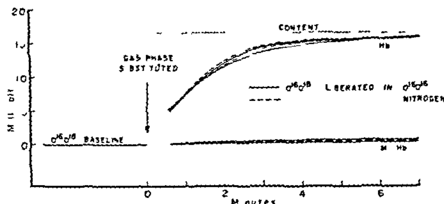


Fig 12 Exchange of oxygen 18 between hemoglobin and gas phase is illustrated by tracings from recorder chart. The ordinate represents amount of tracer liberated from the solutions Oxygen capacity 16 vol per cent.

Results Tracings of the recorder chart are presented in Fig 12 It will be seen that the release of $O^{18}O^{18}$ into oxygen or into nitrogen was identical in rate and amount Inasmuch as we know that the unloading would be complete with nitrogen, it follows from these measurements that all of the oxygen on hemoglobin exchanged with the dissolved oxygen. The total amount of oxygen in the solution was 81 10^{-3} cm^3 of which about 99 per cent was bound to hemoglobin 3 32 per cent of the oxygen was $O^{18}O^{16}$, and knowing the sensitivity of the mass spectrometer (1 6 10^{-6} cm^3 mv^{-1}) the corresponding instrument reading would be 16 8 millivolts This value is indicated in the diagram and it will be seen that the observed amounts compare favorably with the calculated content

These experiments demonstrate that all the sites exchange their oxygen with water and may be potentially available for oxygen transport

FLUX OF OXYGEN THROUGH FULLY OXYGENATED SOLUTION

It was shown that net transfer of oxygen by hemoglobin prevailed only when gradients in oxygenation were maintained between entrance and exit surface (Fig 6) However photometric observations

indicated that a substantial part of the membrane could be almost completely oxygenated, and yet, a full net flux enhancement was obtained Inasmuch as the process is a steady state the oxygen therefore proceeds at full rate through an oxygen saturated layer within the membrane This raises a question of fundamental importance What is the mobility of oxygen in a fully oxygenated hemoglobin solution?

The problem was investigated by determining the flux of oxygen through such solutions by tracer approach

Method and procedures The diffusion cell is shown in Fig 11B The inlet tube of the mass spectrometer could be connected to either of the chambers separated by the membrane, through vaccine rubber stoppers The upper chamber was filled by flushing with nitrogen gas and the lower with a known oxygen nitrogen mixture while the total gas pressure in both was kept at 1 atmosphere The inlet tube of the mass spectrometer was first connected to the lower chamber and the $O^{16}O^{16}$ current was determined The inlet tube was then switched to the upper chamber, and the oxygen gas, enriched with $O^{18}O^{16}$ was slowly introduced into this until the $O^{16}O^{16}$ current equaled that in the lower chamber The oxygen pressures were then very nearly identical on the two sides of the membrane The inlet tube was switched to the lower chamber again and the oxygen 18 accumulation there was followed on the mass spectrometer Hemoglobin solutions of two different *p*H values were used namely *p*H 7 3 and 6 0 The oxygen pressures ranged from 8 5 to 230 mm Hg

At the start of each experiment the upper chamber contained 1 60 to 1 70 atom per cent oxygen 18 the lower oxygen 18 in natural abundance i e about 0 20 atom per cent The membranes were run for about 10 to 15 minutes to obtain a steady flow, and then for another 30 to 40 minutes while the concentration of the isotopes was measured at time intervals

Calculations of total oxygen flux Isotope fractionation effects caused by differences in reaction rates are small and inconsequential in the present context and have been neglected The total oxygen flux through the membranes can easily be derived from the tracer flux in the following way

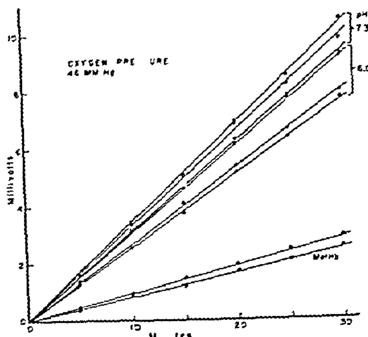


Fig. 13 Examples of the observed tracer data Recorded oxygen 18 accumulation in lower chamber is plotted against time The diagram shows all observations (8 membranes) at one arbitrarily chosen pressure Because of differences in isotope concentrations and instrument sensitivity the curves are not directly comparable Note linearity which shows that the system approximates a true steady stat

The accumulation of $O^{18}O^{16}$ in the lower chamber was recorded in millivolts (mv) (Fig 13) The corresponding *volume* of tracer gas was obtained by multiplying these values by a conversion factor f

$= \frac{V}{S\ P}$ cm³ mv⁻¹ where V is the volume of the chamber (12 8 cm³) S the sensitivity (mv per mm Hg oxygen) and P the atmospheric pressure (760 mm Hg) The sensitivity which was only determined for $O^{16}O^{16}$ (Fig 10) could be directly used for $O^{18}O^{16}$ as no instrumental discrimination between the isotopes was apparent (Table I)

The concentration of $O^{16}O^{16}$ was identical on both sides of the membrane and only the movement of $O^{18}O^{16}$ was observed For small changes in concentration, an approximate correction for back flux of $O^{18}O^{16}$ from the lower chamber can readily be made It rarely amounted to more than 12 to 15 per cent of the accumulation rate

At time zero the concentrations of $O^{18}O^{16}$ in the upper and lower chambers are respectively C_1 and C_2 and the amount of $O^{18}O^{16}$ present in the lower chamber is Q_2 The amount of $O^{18}O^{16}$

entering the lower chamber in a time t is proportional to the concentration of the isotope in the upper chamber

$$\Delta Q_1 = C_1 \; k_1$$

The amount of $O^{18}O^{16}$ leaving the lower chamber is proportional to the concentration of the isotope there

$$\Delta Q_2 = C_2 \; k_2$$

The amount of $O^{18}O^{16}$ in the lower chamber at time t is therefore

$$Q = Q + \Delta Q_1 - \Delta Q_2$$

$$\Delta q = Q - Q = \Delta Q_1 - \Delta Q_2$$

where Δq is the observed change of isotope amount

Assuming that the proportionality factors (permeation constants) k_1 and k are the same in the two directions ($=k$) we have

$$\Delta q = k (C_1 - C)$$

$$= k \; C_1 (1 - \frac{C_2}{C_1})$$

$$= \Delta Q_1 (1 - \frac{C_2}{C_1})$$

$$\text{or} \quad \Delta Q_1 = \Delta q \frac{1}{1 - \frac{C}{C_1}}$$

Therefore by multiplying the observed amount of $O^{18}O^{16}$ by the factor $\frac{1}{1-\frac{C_2}{C_1}}$ the true accumulation of the isotope is obtained

Inasmuch as the concentrations of $O^{16}O^{16}$ and $O^{16}O^{16}$ in the upper chamber remained constant during an experiment the total amount of oxygen which has accumulated in time t is $\Delta Q_1 \frac{C}{C_1} + \Delta Q_1$ when C is the concentration of $O^{16}O^{16}$ in the upper chamber

The total oxygen flux (F) through the membrane is therefore

$$F = K (\Delta Q_1 \frac{C}{C_1} + \Delta Q_1)$$

$$= k \; \Delta q \frac{1}{1 - \frac{C_2}{C_1}} (\frac{C}{C_1} + 1)$$

where K is a proportionality factor dependent upon the time and the geometry of the system. The $O^{16}O^{16}$ concentration being the same on both sides of the membrane, the equation can be written

$$F = K \quad \Delta q \frac{1}{1 - \frac{R}{R_1}} \left(\frac{1}{R_1} + 1\right)$$

where R_1 and R_2 are the ratios of $O^{18}O^{16}$ to $O^{16}O^{16}$ in the upper and the lower compartments respectively

Examples

Fig 13 shows examples of the observed data. The average slope from one of the hemoglobin curves given in the diagram (pH 6.0) is 0.306 mv min^{-1}. The ratio of $O^{18}O^{16}$ to $O^{16}O^{16}$ in the upper chamber was 0.0366 during the experiment while in the lower chamber the ratio increased from 0.0037 to 0.0054, average 0.0045, over the 30 minute time interval. The values for f and K were respectively 1.82×10^{-4} cm^3 mv^{-1} and 1.29×10^{-3} min cm^{-2} sec^{-1}, the latter including a correction for standard temperature 0° C. Hence the total oxygen flux is

$$F = 0.306 \times 1.82 \times 10^{-4} \times 1.29 \times 10^{-3} \frac{1}{1 - \frac{0.0045}{0.0366}} \left(\frac{1}{0.0366} + 1\right)$$

$$= 2.32 \times 10^{-6}\ cm^3\ cm^{-2}\ sec^{-1}$$

Similarly, one of the rates in methemoglobin solution was 0.093 mv min^{-1}. The ratio was 0.0373 in the upper chamber. The ratio was 0.0039 to 0.0043 averaging 0.0041 in the lower chamber. The oxygen flux in this case is

$$F = 0.093 \times 1.82 \times 10^{-4} \times 1.29 \times 10^{-3} \frac{1}{1 - \frac{0.0041}{0.0373}} \left(\frac{1}{0.0373} + 1\right)$$

$$= 0.69 \times 10^{-6}\ cm^3\ cm^{-2}\ sec^{-1}$$

Results. As evident from the basic tracer measurements (Fig 13) the oxygen 18 permeation through hemoglobin solution was substantially increased over permeation through methemoglobin solution. The total oxygen flux through the solutions calculated from the tracer observations is given in Fig 14. It must be emphasized that this oxygen flux does not represent a net transfer through the solutions but is a unidirectional flux component which discloses the *rate at which oxygen passes through the solution in any direction* for there is no oxygen gradient.

Above pressure of full oxygenation (cf Fig 2) the flux enhancement was a constant addition to the rate of simple diffusion obtained

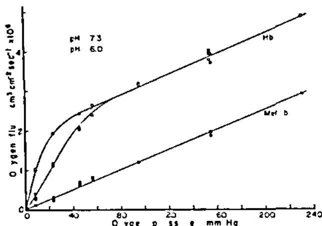


Fig 14 Oxygen flux through hemoglobin and methemoglobin solutions of uniform oxygenation gradient The data are calculated from oxygen 18 accumulation rates Each point represents one membrane Oxygen capacity 20 – 22 vol per cent temperature 25 C

through the methemoglobin solution Thus this effect resembles the net effect (Fig 4) and comparing the two sets of data it is found that the absolute rates in fact are the same within experimental uncertainties While the maximum flux enhancement above averaged 1 9 10^{-6} cm³ cm⁻ sec⁻¹ the net flux enhancement was 1 8 10^{-6} cm³ cm⁻ sec⁻¹ The fluxes through the methemoglobin solution which provides a check of the methods were respectively 0 13 10^{-6} and 0 15 10^{-6} cm³ cm⁻ sec⁻¹ per cm Hg

The fundamental points which these tracer experiments demonstrated are (1) The random oxygen flux was greatly increased in a fully oxygenated hemoglobin solution (2) The enhancement in random flux was equal to the net effect (3) A gradient in hemoglobin oxygenation was not required for accelerating the oxygen transfer through the solution

FLUX OF OXYGEN THROUGH PARTIALLY OXYGENATED SOLUTION

The tracer experiments presented in Fig 14 include measurements below full oxygenation pressures A further examination of the diagram reveals that the enhancement in oxygen flux became dependent on pressure and *p*H in the lower range This suggests a relation to the dissociation curve of the hemoglobin and a comparison of

the *enhancement* with the dissociation curve shows that this is so (Fig 15) At *p*H 6 0, the curves were almost identical, showing that there was a direct proportionality between the enhancement and the amount of oxygen bound to the pigment At *p*H 7 3, the match was less satisfactory, but the discrepancy barely exceeded the accumulated experimental uncertainties This relationship shows that the linear velocity of oxygen i e flux divided by content, was constant and independent of oxygenation In other words the enhancement was the same for all oxygen sites

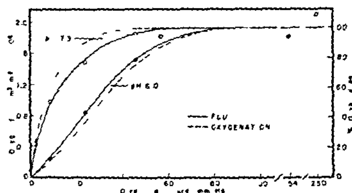


Fig 15 Oxygen flux compared with the oxygenation of hemoglobin at various pressures Flux curves (solid lines) are average of values presented in Fig 14 dissociation curves (stippled lines) are taken from Fig 2

The effect of *p*H evidently is secondary as the flux changes only as the oxygenation of the pigment changes, and above full oxygenation the *p*H does not affect the flux The effect of temperature should be analogous not considering the change in viscosity and thermal motion The temperature aspects have not been fully investigated but checks showed the enhancement above saturation point was not measurably affected by a temperature change of 10° C The insensitivity to temperature furnishes support that the transfer process is of diffusive rather than of chemical nature

FLUX OF OXYGEN THROUGH MEMBRANES OF DOUBLE THICKNESS

It was conceivable that the transfer of oxygen by hemoglobin could be affected in a different fashion than would simple diffusion For instance in a 'bucket brigade mechanism (SCHOLANDER 1960) the

thickness of the membrane strictly speaking would not enter as a rate determining parameter

The relation between thickness and flux was tested as follows Two Millipore filters charged with solution were layered against each other without trapping air between them The excess liquid was blotted off The flux of oxygen through double and single membranes was determined by the tracer method The measurements were made at one oxygen pressure (46 mm Hg) where the full enhancement would be obtained

It will be seen (Fig 16) that the oxygen flux through methemoglobin solution was reduced to half by doubling the membrane thickness The total oxygen flux through the hemoglobin solution reflected the thickness in exactly the same manner Therefore also the hemoglobin mediated flux *per se* (the difference between the curves) is inversely proportional to the length of the diffusion path and in this respect it behaves like simple diffusion

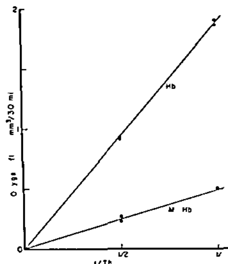


Fig 16 Oxygen flux through single and double membranes Flux is plotted against the inverse of the membrane thickness

Investigations on Myoglobin Solution

Myoglobin differs from hemoglobin in some important respects With one fourth the molecular weight of hemoglobin (SVEDBERG and PEDERSEN 1940) it has a substantially greater molecular mobility It has 1 oxygen combining site per molecule compared to 4 for hemoglobin and also a much higher oxygen affinity (THEORELL 1934 MILLIKAN 1936) These differences invited a study of the transfer of oxygen through myoglobin solution

PREPARATION OF MYOGLOBIN SOLUTION

Myoglobin was obtained from the California sea lion, *Zalophus californianus* The muscles of this animal contain large amounts of myoglobin the meat appears almost black A crude myoglobin extract from a muscle mince rapidly oxidizes to metmyoglobin, therefore special precautions must be taken to obtain a suitable preparation

Muscles from a freshly killed animal were cut into small pieces, massaged and repeatedly washed in saline solution to minimize hemoglobin contamination, and then minced with water The mixture was filtered and muscle globulins were precipitated by basic lead acetate (THORELL 1932 HEMMINGSEN 1963) This stock solution contained the myoglobin in its ferric form (metmyoglobin) Before use portions of the stock solution were converted to the ferrous form by adding sodium dithionite anaerobically through a dialyzing membrane while observing the absorption bands The myoglobin solution was finally concentrated by lyophilization and was adjusted to 9 vol per cent oxygen capacity Capacity analyses were performed as in the hemoglobin solution Hemoglobin contamination of the stock solution was checked by fractional precipitation with ammonium sulphate (THEORELL and DUVE 1947) The precipitates were redissolved in phosphate buffer (*p*H 7 0) and reduced to ferrous form The oxygen and carbon monoxide absorption bands of the final precipitate (myoglobin) the stock solution and a solution of sea lion hemoglobin were examined by a Hartridge Reversion Spectroscope (Table II)

Table II

Mean α-absorption bands of hemoglobin and myoglobin from California sea lion Zalophus californianus Values are in Ångstroms Variations in readings ± 2 Å

	Oxy form	Carboxy form
Hemoglobin	5779	
Myoglobin fresh extract	5810	
Myoglobin stock solution	5808	5778
Myoglobin purified preparation	5808	5777

The absorption bands in the myoglobin preparations were virtually identical This shows that hemoglobin contamination in the stock solution was insignificant (WATSON 1935) The absorption bands were the same as those of a fresh muscle extract indicating that the pigment was not altered by the chemical treatments

NET TRANSFER OF OXYGEN

The net flux of oxygen through myoglobin solution was determined by the volumetric method described on page 17 (Fig 3B) The oxygen (or nitrogen) pressure difference across the membrane was maintained constant at 20 mm Hg while the pressures on both sides were varied from one determination to another

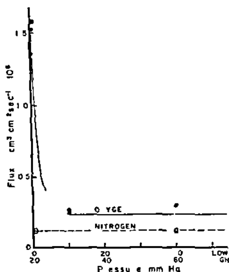


Fig 17 Net flux of oxygen and nitrogen through myoglobin solution The abscissa represents pressures in the two chambers with constant pressure differentials across the membrane 3 membranes Oxygen capacity 9 0 vol per cent temperature 25 C

The results are given in Fig 17 With oxygen pressures higher than 10 mm Hg on the lower side of the membrane the oxygen net flux was about twice that of nitrogen which indicates that the oxygen proceeded through the solution by a simple diffusion However, when the oxygen pressure on the lower side was zero the oxygen net flux increased six fold while the corresponding nitrogen pressures gave an unchanged nitrogen net flux In basic respects therefore the myoglobin data are very similar to those obtained with hemoglobin (Fig 6) and show that the myoglobin increases the rate of net transfer of oxygen provided the solution is not opposed by back pressures of the gas

FLUX OF OXYGEN THROUGH OXYGENATED SOLUTION

The flux of oxygen through fully oxygenated myoglobin solution was determined by the oxygen 18 tracer method (p 25) The experimental procedure was identical to that used for hemoglobin The reference diffusion rate was obtained by metmyoglobin solution

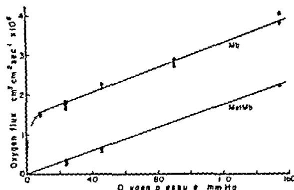


Fig 18 Oxygen flux through myoglobin and metmyoglobin solution of uniform oxygenation The data are calculated from oxygen 18 accumulation rates Each point represents one membrane Oxygen capacity 9 0 vol per cent temperature 25 C

The results (Fig 18) showed a greatly accelerated flux through the myoglobin solution Analogous to the hemoglobin data (Fig 14) the increase in flux appeared as a constant addition to the rate of simple diffusion As would be expected from the high oxygen affinity of myoglobin the full effect was maintained down to the lowest pressure tested (8 5 mm Hg) at which the flux was increased some 15 times This flux enhancement averaged 1 6 10^{-6} cm^3 cm^{-2} sec^{-1} as compared to a net flux enhancement of 1 3 10^{-6} cm^3 cm^{-} sec^{-1} (Fig 17) For this high affinity solution the net flux enhancement would be extremely sensitive to minute back pressures but nevertheless, there was a fair agreement in rates

The enhancement was almost the same as that obtained for *hemoglobin* solution of more than double the oxygen capacity Since there was a good match in the rates of simple diffusion (0 14 10^{-6} and 0 13 10^{-6} cm^3 cm^{-2} sec^{-1} per cm Hg respectively) the enhancements can be directly compared Relating them to the molecular weights of myoglobin and hemoglobin, which are about 17 000 and 68 000 it is found that the rates were roughly inversely proportional to the square root of the molecular weights Thus the differ-

ence in the rates appears to be a result of the difference in the thermal motion of the pigment molecules

Supplementary Investigations

OXYGEN TRANSFER THROUGH HAGFISH HEMOGLOBIN SOLUTION

The hemoglobin of hagfish has a molecular weight of about 20,000 (SVEDBERG and PEDERSEN 1940 ZUCKERKANDL JONES and PAULING 1960) contains 1 heme per molecule and has a half saturation pressure of 3 to 4 mm Hg at room temperature (MANWELL 1958) Thus, the pigment is in many respects similar to myoglobin Blood from California hagfish *Polistotrema stouti*, was pooled and the cells washed with saline and hemolyzed The oxygen capacity of the final solution was 7 0 vol per cent The rate of oxygen transfer through the solution was determined by the volumetric method (Fig 3B)

The results (Fig 19) show that this pigment also gave a substantial enhancement in the oxygen net flux when the oxygen pressure on the lower side of the membrane was zero However the increase in flux was far less than that obtained with myoglobin and this was most likely due to the rather high viscosity of the solution as well as the lower oxygen capacity

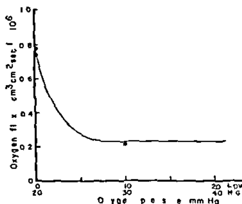


Fig 19 Net flux of oxygen through hagfish hemoglobin solution 2 membranes

OXYGEN TRANSFER THROUGH HEMOCYANIN BLOOD

Inasmuch as the increase in oxygen transfer by hemoglobin and myoglobin appeared to be a direct function of their molecular weights it was of interest to establish whether a pigment of much larger size would give any effect Octopus hemocyanin *(Octopus vulgaris*

and *Eledone mochata)* has a molecular weight of about 2,800 000 (SVEDBERG and PEDERSEN 1940) Blood from a near related species, *O bimaculatus* was concentrated to half volume by dialyzing it against a high molecular carboxy methyl cellulose, Cellugel (PALMSTIERNA 1960) After concentrating an oxygen capacity of 6 2 vol per cent was obtained This analysis was made with the Roughton-Scholander syringe method liberating oxygen by sodium cyanide (REDFIELD, COOLIDGE and HURD 1926 COOKE 1928)

The rate of oxygen transfer through the solution was determined by tracer approach However because hemocyanin still retains its oxygen combining property in its cupric form, it was necessary to use a double tracer procedure to obtain a reference flux A small amount of argon (about 1 vol per cent of the total gas) was mixed with the oxygen 18 enriched oxygen nitrogen mixture in the upper chamber (Fig 11B) the partial pressure of oxygen being the same on both sides of the membrane (46 mm Hg) At this pressure the hemocyanin was 85 per cent oxygenated The accumulation of both tracers in the lower chamber was determined by the mass spectrometer (Fig 20)

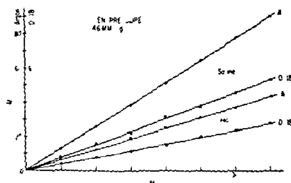


Fig 20 Tracer data used for calculating relative diffusion rates of oxygen 18 and argon through hemocyanin blood Recorded accumulation of the gases plotted against time 2 membranes

The amount of tracer gas ($\triangle q$) which accumulated in 30 minutes was obtained from the mass spectrometer readings by $\triangle q = \frac{\triangle E}{E_1 - E}$ s where E_1 is the average voltage reading for the tracer in the upper chamber E is the corresponding reading in the lower chamber $\triangle$ E is the increase in voltage in the lower chamber over 30 minutes and *s* is a constant factor chosen

arbitrarily as 100 The sensitivity of the mass spectrometer was constant

The relative fluxes of oxygen (i e oxygen-18) and argon through hemocyanin blood are given in Table III

Table III

Fluxes of oxygen and argon through saline and hemocyanin blood

	Relative flux vol /time		$\frac{\Delta q_O}{\Delta q_A}$
	Oxygen 18 (Δq_O)	Argon (Δq_A)	
Saline (0 85 %)	3 28	4 01	0 82
	3 06	3 81	0 80
Hemocyanin	1 62	1 96	0 83
	1 61	2 05	0 79

It will be seen that the oxygen flux as well as the argon flux was substantially less through hemocyanin blood than through saline However the oxygen argon flux ratio was the same for the two solutions and hence the rate of oxygen transfer was not specifically affected by the hemocyanin the gas proceeding through the solution exclusively by simple diffusion The results imply that the molecular motion of the pigment is too small to provide any detectable increase in the oxygen flux

OXYGEN TRANSFER THROUGH ERYTHROCYTES

The question which naturally arises is whether or not the hemoglobin in erythrocytes may significantly increase the oxygen transport Such facilitation effect although small was reported by SCHOLANDER (1960) and has recently been discussed by others (LA FORCE and FATT 1962 SIRS 1963) A quantitative investigation of this problem is difficult but a crude estimation was feasible with the present techniques

Human erythrocytes washed in saline were smeared with a soft brush on top of a Millipore filter grade HA which had been soaked in saline The membrane was placed in the diffusion cell (Fig 11B)

and the oxygen and argon fluxes were determined as described for hemocyanin

In 2 experiments the ratio of oxygen to argon was 1 23 and 1 19, as compared to 0 84 ± 0 04 for saline and methemoglobin solution (Table IV) A very slight hemolysis was apparent when the membranes were soaked in saline after the measurements, but this was so small that it would hardly affect the results The nearly 50 per cent increase in oxygen flux relative to argon flux indicates that the hemoglobin molecules in the erythrocytes possess a certain mobility and potentially could facilitate the oxygen net transport

DIFFUSION OF ARGON AND OXYGEN THROUGH SALINE AND HEMOGLOBIN SOLUTIONS

The accelerated transfer of oxygen by hemoglobin as well as the rate of simple gas diffusion is markedly affected by the hemoglobin concentration (SCHOLANDER 1960, LONGMUIR and ROUGHTON 1952

Table IV

Relative fluxes of oxygen and argon through saline and hemoglobin solutions The methemoglobin solution was of the same concentration as the strongest hemoglobin solution

	Relative flux vol /time		$\frac{\Delta q_O}{\Delta q_A}$	$\frac{(\Delta q_A)\ sample}{(\Delta q_A)\ saline}$
	Oxygen 18 (Δq_O)	Argon (Δq_A)		
Saline (0 85 %)	3 28	4 01	0 82	1 00
	3 06	3 81	0 80	
Methemoglobin	*2 30*	*2 61*	*0 88*	*0 67*
	2 28	2 71	0 84	0 69
Hemoglobin	7 16	3 61	1 99	0 92
(10 %, O capacity)	7 05	3 52	2 00	0 90
Hemoglobin	7 98	2 83	2 82	0 72
(21 %, O capacity)	8 42	2 90	2 91	0 74

PIRCHER 1952) In the course of this study, data on this effect have accumulated and these are presented in Table IV The determina-

tions were made by the double tracer method described for the hemocyanin experiments

Discussion

The experimental data showed that hemoglobin and myoglobin increased the random oxygen flux in a solution and that this flux was identical to the net flux obtained when one side of the membrane was maintained at zero oxygen pressure The accelerated transfer rates in the two cases therefore are different aspects of the same basic transfer process

This process depends on the mobility of the pigment molecules This was borne out by the retarding effect of viscosity and more revealingly, by the decrease in oxygen flux by increasing molecular size Thus the pigment molecules undoubtedly act as carriers for the oxygen and thereby provide an additional pathway through the solution

The observed oxygen flux always appeared as a sum of two separate components namely (1) a simple Fick s diffusion of the gas through the liquid, and (2) a transfer by the pigments which was independent of the oxygen pressure over a wide range This suggests two things One is that an isolation exists between the oxygen which is carried by the pigment and that which proceeds by diffusion through the liquid The other is that the rate of transfer by the pigment is independent of the oxygenation gradients through the solutions It must be emphasized however that the two flux components cannot be measured separately and simultaneously and the apparent maintenance of Fick s diffusion therefore could be spurious

Isolation of pathways The oxygen in physical solution readily exchanges with that combined with hemoglobin (Fig 12) It is therefore only in a restricted sense that the two categories of oxygen can be considered independent of each other

When there are no gradients across the solution and equilibrium exists the presence of oxyhemoglobin (or oxymyoglobin) does not affect the concentration of dissolved oxygen as compared to that in a met solution (i e methemoglobin or metmyoglobin solution) Con

sequently, the rate of self diffusion of *dissolved* oxygen would remain the same That is an equal number of oxygen molecules traverse a given distance in the water per time unit, but any of these molecules may have been exchanged with those on the pigment

Such an equilibrium situation obtained in the tracer flux experiments, where there was no mass flow of oxygen Although a gradient of oxygen 18 was maintained for tracing the movement of the oxygen, the oxygen flux was calculated on the basis of total oxygen involved As expected, the flux enhancement provided by the pigment showed up as a component in excess of the rate of simple diffusion (Figs 14 and 18) The flux enhancement can here be considered as a separate process, as it did not affect and evidently was not affected by, the diffusion of oxygen through the liquid

With an oxygen gradient (Fig 4) the net flux was equal to the preceding random flux, and again appeared to be a sum of two components This similarity, as well as the identity in rates, suggests that the oxygen net flow did not change the relation between dissolved and combined oxygen, the separation of the two pathways appears to be maintained in the net transfer process

The oxygen tension gradient When oxygen diffuses through water or methemoglobin solution across which there is a constant oxygen tension difference the drop in oxygen concentration is the same for all equal segments along the path, i e, the tension gradient is linear

Inasmuch as the additional oxygen flux provided by the pigments evidently did not affect the concentration of dissolved oxygen, and the rate of simple diffusion was the same as in a methemoglobin solution the tension gradient should be linear also when net transfer prevails

The pigment oxygenation gradient If complete chemical equilibrium existed between the oxygen and the pigment along a linear tension gradient the gradient in pigment oxygenation would be non linear and should in fact follow the dissociation curve as derived from the linear tension drop

Direct observations by photometric methods (p 14) showed that the oxygenation of hemoglobin in the membrane was, indeed, non linear with oxygen pressures which were well above saturation

point at the entrance surface However, the oxygenation lagged with respect to conditions of complete equilibrium Therefore provided the tension gradient was linear there was a deficit in potential oxygenation along the gradient

Transfer in relation to pigment oxygenation The net flux enhancement (Fig 4) reached maximum value when the oxygen pressure at the entrance surface was equal to the saturation pressure of the solution For all pressures higher than this the enhancement remained the same But the oxygenation inside the membrane increased with increasing pressures (Evans 1964) and consequently the enhancement was constant for different oxygenation gradients

This and the lack of equilibrium inside the membrane are compatible with the relationship between random flux and oxygenation (Fig 15) only if the transfer process is a function of the oxygenation at the interfaces and is independent of that inside the membrane This concept is of particular significance It implies that the molecular events may not be fully described by a conventional diffusion approach (Evans 1964) as this requires that the flux be proportional to the gradient between the boundaries

The fact that the enhancement in random flux so closely reflected the equilibrium oxygenation of hemoglobin (Fig 15) suggests that (1) equilibrium or nearly so existed between hemoglobin and oxygen and between solution and gas phase at the entrance surface, and that (2) the transfer process was not rate limited by the oxygenation reactions If this had not been the case a delay would be manifested in the flux that is the flux curve would be displaced towards higher pressures relative to the dissociation curve

With carbon monoxide which has a much slower dissociation rate than oxygen from hemoglobin a substantial difference was indicated between the equilibrium saturation and the net flux (Mochizuki and Forster 1962) At low pressures a fifteen fold flux enhancement was observed which was only one tenth of that expected without limitation by the reaction rates Such limitation was supported by the fact that the presence of oxygen which would increase the dissociation rate f carbon monoxide also increased the flux enhancement

Transfer in relation to back pressures Full net flux enhancement required zero oxygen pressure at the exit surface (Figs 6 and 17) When the oxygen pressure here exceeded the equilibrium saturation pressure the enhancement was completely abolished This suggests

that the enhancement was determined by the difference in hemoglobin oxygenation between the two surfaces.

But it must be emphasized that oxygenation *per se* does not block the transfer system as oxygen did proceed through a saturated solution at undiminished rate. Hence, the abolishment of the net effect by back pressures was clearly a result of a cancelling return flux, rather than inhibition of the transfer system itself.

Transfer in relation to pigment diffusion. The relationship between flux enhancements and the molecular weights of hemoglobin and myoglobin (p. 34) suggested that the transfer by the pigments was a direct function of their molecular mobility. It is therefore of interest to compare the linear velocities of oxygen with the actual diffusion rates of myoglobin and hemoglobin.

The linear velocity (v) per unit membrane area (cm^2) is obtained from the flux and oxygen content, i.e. $v = \frac{F \cdot x}{C}$, where F is the oxygen flux (cm^3 cm^{-2} sec^{-1}), C is the oxygen content (cm^3 cm^{-3}), and x is the thickness (cm). We shall consider the flux at an oxygen pressure where the solutions were fully oxygenated, namely, 100 mm Hg, or 0.131 atm.

The tracer experiments (Figs. 13 and 18) gave enhancements by hemoglobin and myoglobin of respectively 1.9 × 10^{-6} and 1.6 × 10^{-6} cm^3 cm^{-2} sec^{-1} and through the corresponding met solutions the fluxes were 1.3 × 10^{-6} and 1.4 × 10^{-6} cm^3 cm^{-2} sec^{-1}. If an oxygen solubility of 0.029 cm^3 cm^{-3} atm^{-1} (PIRCHER 1952) is used for the met solutions, the ratio between the linear velocity of oxygen transferred by hemoglobin (C = 0.20 cm^3 cm^{-3}) and by simple diffusion is

$$\frac{(v_O)_{Hb}}{(v_O)_{MetHb}} = \frac{1.9 \times 10^{-6}}{0.20} \cdot \frac{(1.3 \times 10^{-6})^{-1}}{(0.029 \times 0.131)^{-1}} = 0.028$$

The thickness is constant and will not affect the ratio. The corresponding ratio for myoglobin solution (C = 0.09 cm^3 cm^{-3}) is

$$\frac{(v_O)_{Mb}}{(v_O)_{MetMb}} = \frac{1.6 \times 10^{-6}}{0.09} \cdot \frac{(1.4 \times 10^{-6})^{-1}}{(0.029 \times 0.131)^{-1}} = 0.048$$

The ratios between the diffusion velocities of the pigments and the oxygen are obtained by means of the diffusion coefficients. Although these are not known for the present system, the values established for the solution of lower concentrations (POLSON 1939) may be used. For 3.8 per cent hemoglobin solution the diffusion coefficient (D_{Hb}) is 6.83 × 10^{-7} cm^2 sec^{-1} and for 0.4 per cent myoglobin solution D_{Mb} is 11.26 × 10^{-7} cm^2 sec^{-1}. The diffusion coefficients for oxygen in methemoglobin solution (4.4 per cent) and water are 2.11 × 10^{-5} and

2 30 10 ⁵ cm² sec ¹ respectively (PIRCHER 1952) The ratio between the diffusion coefficients therefore becomes

$$\frac{D_{Hb}}{D_O} = \frac{6\ 83 \quad 10^{7}}{2\ 11 \quad 10^{5}} = 0\ 032$$

$$\frac{D_{Mb}}{D_O} = \frac{11\ 26 \quad 10^{7}}{2\ 30 \quad 10^{5}} = 0\ 049$$

The ratios of the diffusion coefficients in each of the solutions are almost identical to those obtained for the linear velocities of oxygen It follows that the linear velocities for the enhancements relate to the diffusion coefficients of the pigments as does the linear velocity for the diffusive flux to the diffusion coefficient of the gas This strongly indicates that the transfer process is rate limited by the spatial movement of the oxyhemoglobin (and oxymyoglobin) molecules the flux being proportional to the diffusion coefficient

Molecular transfer mechanism To summarize some principal characteristics of the oxygen transfer process the following major points may be derived from the data

1 The enhancements in random flux and net flux are the same and thus the transfer process does not depend on oxygenation gradients
2 The enhancement in random flux is directly proportional to the pigment oxygenation in equilibrium indicating that the transfer process is not rate limited by the oxygenation reactions
3 The enhancement is proportional to the diffusion velocities of the pigments and is evidently rate limited by this
4 The enhancement is inversely proportional to the thickness of the membranes
5 The enhancement in net flux depends on the maintenance of a difference in pigment oxygenation between the surfaces but is independent of the amount of oxygenated pigment in the membrane

In many basic respects the transfer process is of a diffusive nature and may *numerically* be described by diffusion equations If we consider the total net flow of oxygen per unit area through a hemoglobin solution the steady state flux (F) may be expressed by the following empirical equation

$$F = a \quad D_O \frac{(C_O)_1 - (C_O)}{X} + a \quad D_{Hb} \frac{(C_{OHb})_1 - (C_{OHb})_2}{X}$$

where $(C_{O_2})_1$ and $(C_{O_2})_2$ are the concentrations of dissolved oxygen at the interfaces, $(C_{OHb})_1$ and (C_{OHb}) are the corresponding concentrations of oxygenated hemoglobin, x the thickness, D_{O_2} and D_{Hb} the diffusion coefficients of oxygen and hemoglobin respectively, and a an empirical proportionality factor *The first term* represents the diffusive flux through the liquid, the second the hemoglobin mediated flux

This relationship, which strongly suggests a 'carrier' type mechanism, virtually excludes any interface or orientation phenomena as possible causes of the effect Two mechanisms which comply with this concept have been proposed One is based on agitation of the hemoglobin molecules with transfer of oxygen on collision This system may be visualized as a chain of hemoglobin molecules through which the oxygen is transmitted from one end to the other *in a 'bucket brigade fashion* (SCHOLANDER 1960) A related concept has been elaborated on and given a more precise formulation (EVANS 1964) Rate-limiting factors here would be the frequency of collisions, which is proportional to the diffusion coefficient and the completeness of exchange on collision The other mechanism, discussed by several authors (COLLINS 1961a b, WANG 1961, 1963, FATT and LA FORCE 1961 1962 MOCHIZUKI and FORSTER 1962), is based on diffusion of oxyhemoglobin molecules and equations for such a process were proposed A model has not been formulated but the concept implies that oxygen combines with the hemoglobin molecules at one place moves with them to another, and there dissociates off into the liquid again The spatial diffusion of the pigment molecules would here enter as a rate-limiting factor

For both mechanisms, important aspects to consider are the spacing of the hemoglobin molecules their diffusion velocity and the lifetime of the oxyhemoglobin complex The linear dimensions of space occupied by the hemoglobin molecules can be estimated from Avogadro s number and the molar concentration of hemoglobin This comes out at about 10^{-6} cm or 100 Ångstrom, which is about twice the diameter of the molecules themselves (PERUTZ *et al* 1960) i e the space between molecules is 50 Ångstrom From the diffusion coefficient of hemoglobin in 3 8 per cent solution which is 6 83 10^{-7} cm sec^{-1} (POLSON 1939) the transit time of the molecules for the 0 015 cm thick membranes is 3 3 10^{3} sec Compared with this the time for the combination of oxygen is extremely short,

10^{-} to 10^{-3} sec (Roughton 1959) Assuming that there is an ideal dynamic exchange which indeed was indicated in Fig 12 the lifetime of the oxyhemoglobin complex would be of the same magnitude Hence oxygen would remain on hemoglobin only for a distance of a few Ångstroms say one tenth the distance of spacing This indicates that the oxygen molecules are transmitted through the system in steps that are far shorter than the mean free path of the hemoglobin molecules

Transfer of oxygen by oxyhemoglobin diffusion as suggested by the several authors above is compatible with the kinetics of hemoglobin and its reactions with oxygen The flux enhancement was proportional to the difference in oxygenation at the surfaces which, however does not necessarily justify the application of Fick s law for describing the molecular mechanism involved Fick s first law requires that under steady state conditions the concentration drop through a homogenous medium must be linear that is $\frac{dc}{dx}$ must be constant (Jost 1960) In the present case c represents the concentration of oxyhemoglobin but as shown this gradient is far from linear Consequently, the proportionality factor obtained the oxyhemoglobin diffusion coefficient, would have no defined relationship to the molecular velocity of the pigment as it would be different for various segments of the diffusion path No provisions have been made for this in the proposed equations Thus the diffusion concept as it is worked out for the movement of a single component is hardly applicable to this case of two interacting components

The problem with the gradient as well as other difficulties inherent in the diffusion approach led Enns (1964) to suggest an alternative treatment which is based on the theory of heat transfer It is postulated that the oxygen like diffusion of heat is carried on by exchange when hemoglobin molecules collide Independent of the gradient the transfer mechanism is there in that the hemoglobin molecules pass on the collisions in any direction In this mechanism the oxygen flux would be determined by the oxygenation at the entrance and exit surfaces The diffusivity coefficient of hemoglobin was extrapolated on a molecular weight basis from that of water Using this value and assuming that oxygen transfer on collision is complete the theoretical transfer rate obtained agreed with empirical data

An important aspect of both this system and the bucket brigade system needs clarification Would there be a net gain by collision exchange if the hemoglobin molecules freely exchange their oxygen with the liquid between collisions? Strictly speaking, the heat transfer analogy would hold only if the energy, i e, the oxygen, is not dissipated from the molecules between collisions

In conclusion, therefore the theories which have been proposed for the oxygen transfer process are insufficient to describe the *molecular* events But presently, it appears difficult to advance the theory decisively There are obvious gaps in empirical information concerning some of the fundamental parameters involved In particular it would be desirable to obtain more exact knowledge about the pressure and oxygenation gradients, and how these relate to equilibrium conditions Also it would be of great value to know the diffusion rate of the pigments in the experimental membranes

General implications Biological transport processes provide many examples of facilitated diffusion in which carriers appear to be involved Although the present oxygen hemoglobin system undoubtedly represents only one of several types of mechanisms this system may perhaps be similar to others in particular those involving non-electrolytes (BOWYER 1957 BRITTON 1964) It is outside the scope of this investigation to deal further with this extensive field But inasmuch as the carrier in no previous case has been isolated or identified it may be of interest briefly to generalize some of the information obtained in the present case

If the carrier is free in a medium and moves solely by its thermal *motion* the following provisions apply

1 The randomness of the simple diffusion of both the carrier and the substance can be fully maintained
2 The reaction between substance and carrier must be rapid compared to the transit time A difference in loading and unloading rates is not required
3 The product of the diffusion velocity of the carrier and the amount of substance combined with it should at least be of magnitude comparable to that of the diffusion velocity of the substance and the dissolved content
4 The concentration of substance on the exit side must be lower than that which will saturate the complex in equilibrium

Physiological implications One may naturally wonder if the transport enhancement provided by hemoglobin and myoglobin in solution may be of physiological significance to the organisms

Hemoglobin in higher animals is restricted to the erythrocytes and the problem of possible transport facilitation by this pigment would be limited specifically to the passage of oxygen into and out of these cells An increase in the net transport of oxygen here would depend on the fulfillment of two requirements the hemoglobin molecules must possess a spatial mobility and the oxygen pressures

must be such that an adequate difference in oxygenation is maintained between the inside and the outside of the cells

Experimentally a slight but significant flux enhancement was obtained when oxygen was diffused through layers of erythrocytes This would indicate that the hemoglobin retains a certain mobility but compared to the moderately concen trated solutions investigated it would be much less However recent diffusion studies of erythrocyte suspensions with a non steady state spectrometer method have discounted transport facilitation of oxygen in the erythrocytes (SIRS 1963) These contradicting results may not be of physiological relevance anyway since it is doubtful that the oxygenation differentials *in vivo* ever become sufficiently large to provide a net transport increase of any importance

An interesting idea for transport facilitation of oxygen in the erythrocytes suggested by CROSBY (1957) should be mentioned The spherical hemoglobin molecules with the heme groups near the surface could spin like inking rollers of a printing press pressing oxygen from one molecule to the next through a chain of hemoglobin molecules The spacing of the hemoglobin seems to be sufficient for a free rotation (BATEMAN HSU KNUDSEN and YUDOWITCH 1953) However such a mechanism may be quite different from the transfer by the hemoglobin in solution and hence would not necessarily depend on the same factors

The function of myoglobin found in various amounts in muscle cells is not so clear as that of hemoglobin. Evidently it constitutes an intermediary link be tween the oxygen supplied by the blood and the metabolic processes in the cells One function undoubtedly is to act as a transient store for oxygen smoothing out brief fluctuations in oxygen demand (MILLIKAN 1937) The tissues of a number of invertebrate animals contain substantial amounts of hemoglobin and the func tion of the pigment here may be analogous to that of myoglobin in higher organ isms

The question of whether myoglobin in addition to storing oxygen may more directly promote its transport in the cells has not been investigated for *in vivo* situations BARCROFT (1934) and MILLIKAN (1939) discussed the various roles that myoglobin might have but they found it unlikely that the pigment would be of any aid to diffusion in a system of continuous oxygen flow But from the present information this would again depend on the prevailing oxygen tension the mobility and distribution of the pigment in the cells and of course on the pigment concentration

The most efficient transport facilitation would occur when the myoglobin is maintained saturated at the point of entry and is reduced at the point of oxygen demand The tension situation in muscle cells seems to favor this requirement MILLIKAN (1937) observed spectrometrically a complete reduction of myoglobin during the contraction of a muscle

The concentration of myoglobin varies greatly from one animal to another as it does among various muscles in the same animal Large concentrations are found in diving mammals muscles of seals commonly have an oxygen capacity of about 5 vol per cent (SCHOLANDER 1940) The myoglobin content in other animals is usually much less A common value for heart muscles is 0 5 g myoglobin per 100 g muscle (WATSON 1935 BIORCK 1949 LAWRIE 1952) corresponding to an oxygen capacity of about 0 7 vol per cent Since the transfer enhancement was roughly

proportional to the pigment concentration (Table IV and SCHOLANDER 1960) one finds by extrapolation that a solution of only 0 7 vol per cent oxygen capacity could give an enhancement of at least 50 per cent

Little or no information is available regarding the mobility and the distribution of myoglobin in the cells If the distribution of the pigment is uniform throughout most of the cell it is likely that its mobility would be restricted compared with that in the present solutions and a possible enhancement would be correspondingly less It would therefore be premature to assume that the particular effect described in the present investigation plays any role in the organisms The problem must await clarification from direct *in vivo* determinations

Summary

The purpose of this investigation has been to elucidate the role of hemoglobin (human) and myoglobin (sea lion) in the steady state transfer of oxygen through solutions of the pigments Thus, convection free layers were obtained by suspending the solutions in porous membranes (Millipore filters) and the transfer of oxygen through these was determined by volumetric and by oxygen 18 tracer methods

The main facts revealed by the data were

1 The net transfer of oxygen through both hemoglobin and myoglobin solutions was greatly accelerated compared with the rate of simple oxygen diffusion provided the pressure on the exit side was low enough to desaturate the pigments Back pressures of oxygen completely abolished the net effect (Figs 4 6 and 17)
2 The transfer enhancement always showed up as a numerical addition to the rate of simple diffusion given by a linear oxygen tension gradient, and it was constant for all pressures higher than saturation point on the entrance side
3 Over the pressure range where the enhancement was constant, the oxyhemoglobin content in the membranes varied, and it was substantially increased relative to that of a linear oxyhemoglobin gradient (p 14)
4 Experiments with membranes maintained between equal oxygen pressures but different oxygen 18 concentrations showed that the oxygen transfer in any direction was always increased at a rate which was proportional to the pigment oxygenation

The increase was constant above saturation point and had the same characteristics as the net effect The random flux enhancement was identical to the net flux enhancement (Figs 14 15 and 18)

5 The exchange of oxygen between oxyhemoglobin and liquid was fully maintained at all degrees of oxygenation (Fig 12)

6 The rate of oxygen transfer was also increased through hag fish hemoglobin solution and erythrocytes while no effect was obtained with octopus hemocyanin (pp 35–38)

Evaluations of the empirical data further established the following characteristics

7 Increase in oxygen transfer did not require a pigment oxygen ation gradient, as (a) the net transfer proceeded through a substantially saturated layer within the membrane and (b) the random flux was identically increased without oxygenation gradients

8 The net effect was constant for different pigment oxygenation gradients within the membranes

9 The linear velocity of oxygen (i e flux divided by content) proceeding via hemoglobin was constant and independent of the pigment oxygenation and hence the transfer process was not rate limited by the oxygenation reactions

10 The enhancement was proportional to the diffusion rate of the pigments (hemoglobin myoglobin) and was rate limited by this

11 It is concluded that the molecular aspects of the transfer process are insufficiently described by the equations for simple diffusion The process may involve other concepts

12 The potential physiological significance of the transfer effect is briefly discussed It is doubtful that the effect plays any major role *in vivo*

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EVIDENCE FOR THE EXISTENCE OF MONOAMINE NEURONS IN THE CENTRAL NERVOUS SYSTEM

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IV Distribution of monoamine nerve terminals in the central nervous system

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II

Experimentally Induced Changes in the Intraneuronal Amine Levels of Bulbospinal Neuron Systems

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ANNICA DAHLSTRÖM and *KJELL FUXE*

INTRODUCTION

The cellular localization of monoamines in the spinal cord of mouse and rat has been studied using a histochemical fluorescence method for the demonstration of CA[1] and 5 HT in combination with a pharmacological approach and transection experiments (CARLSSON FALCK FUXE and HILLARP 1964) It was found that NA and 5 HT are abundantly accumulated in the synaptic terminals that derive from special systems of nerve tracts descending in the lateral and anterior funiculi probably from supraspinal centres The significance of this is enhanced by the discovery of new kinds of nerve cells localized to certain regions in the lower brain stem and containing small amounts of DA NA or 5 HT (DAHLSTRÖM and FUXE 1964*a*)

A large nigro neostriatal DA neuron system has now been described in detail (ANDEN CARLSSON DAHLSTRÖM FUXE HILLARP and LARSSON 1964 ANDEN DAHLSTRÖM FUXE and LARSSON 1965) and certain other large ascending systems of CA neurons from the lower brain stem have also been discovered (DAHLSTRÖM FUXE OLSON and UNGERSTEDT 1964)

In the present paper it will be shown that the special systems of nerve tracts in the spinal cord belong to several different bulbospinal NA and 5 HT neuron systems which will be mapped out in detail with the help of the changes in the intraneuronal amine levels that occur after axotomy alone or axotomy in combination with MAO inhibition

[1] *Abbreviations used* CA catecholamine(s) DA = dopamine NA = noradrenaline A = adrenaline 5 HT = 5 hydroxytryptamine 5 HTP = 5 hydroxytryptophane DOPA = 3 4 dihydroxyphenylalanine MAO = monoamine oxidase

[2] The names of the monoamine cell groups in the medulla oblongata are the same as introduced in the study by DAHLSTRÖM and FUXE 1964*a*

MATERIAL AND METHODS

The spinal cord was studied at different levels in about 525 male Sprague Dawley rats (body wt 200 to 250 g about 2 months old) A number of normal mice guinea pigs rabbits cats and dogs were also used

A large number of different kinds of lesions were made in the spinal cord of the rat (see Table I and Textfig 1a–g) The vast majority of the total transections were made at C8 but some also at Th4 and Th12 Half of the partial transections were made at C4 half at C8 Half of all the operated animals with lesions in the spinal cord were treated with the potent MAO inhibitor nialamide (500 mg/kg i p) 5 to 6 h before killing All operations were performed under ether anaesthesia and with a posterior approach An incision was made above the vertebrae the muscles were divided and the ligaments between two vertebrae and the dura mater transected Under a dissection microscope the respective parts of the spinal cord (see Textfig 1) were cut with part of a razor blade except for the total transections which were made with a fine pair of scissors In the animals in which the spinal roots were to be cut 3 to 4 vertebrae arches were removed and the roots reached by a gentle pushing of the spinal cord and cut with part of a razor blade Both the cutting of the roots and accidental injuries made directly on the spinal cord were always checked in the serial sections made by examination in the fluorescence or light microscope The animals with a total transection were kept in good condition by daily injections of 5 ml of a 5 5 % glucose solution i p 5 ml of an amino acid glucose solution (Aminosol Glycose Vitrum) s c and 10 000 I U of penicillin (Suspenin Vitrum) i m The bladder was emptied twice daily and the animals kept at a room temperature of +23–+25 C

15 young Sprague Dawley rats (b wt 100 g 4 weeks old) were also used in half of them the cord was totally transected at C8 while in the rest a transection of half the cord was made at C4 They were killed 2 or 4 days after operation

Nialamide treatment was performed also in normal animals (50) in the same way as described for the operated animals and in animals (25) previously pretreated with reserpine (10 mg/kg i p 24 h before killing) Reserpine treatment was performed in the same way in 30 normal rats and

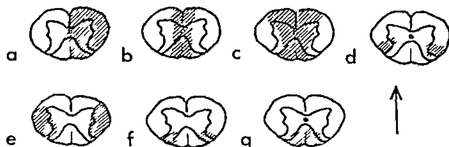
TABLE 1 *Time before killing after transection of the spinal cord*

	Days (Number of animals in parentheses)								
Total transection	1/4(6)	1(12)	2(10)	3(16)	4(8)	6(10)	8(16)	10(12)	12(8)
Lesion of one half of the spinal cord (see Textfig 1*a*)	1(4)	2(7)	4(11)	8(9)	12(6)	16(10)	19(12)	40(6)	
Bilateral lesion of the anterior funiculus (see Textfig 1*b* and *c*)	1(3)	2(8)	4(14)	10(8)	16(1[illegible])	19(10)			
Bilateral lesion of the dorsolateral part of the lateral funiculus (see Textfig 1*d*)	2(8)	4(5)	8(7)	16(6)	19(10)				
Bilateral lesion of the lateral funiculus (see Textfig 1*e*)	1(4)	2(8)	4([illegible])	8(5)	16(11)	19(9)			
Bilateral lesion of the posterior horn (see Textfig 1*f*)	2(6)	4(5)	8(4)	16(8)					
Bilateral lesion of the posterior funiculus (see Textfig 1*g*)	2(6)	8(4)							
Bilateral lesion of the spinal roots belonging to cervical segments 5 to 8	10(8)								
Bilateral lesion of the spinal roots belonging to thoracical segments 4 to 7	35(9)								

in 5 rats which were totally transected at C8 2 days before administration of the drug 5 other rats operated on in this way were administered reserpine (10 mg/kg i p) 4 h before the killing

A low dose of malamide (50 mg/kg) was administered to 10 rats 5 to 6 h before killing 5 of these rats had 12 days before been totally transected at C8

α methyl meta tyrosine treatment (400 mg/kg i p) was performed in 12 normal rats 24 h before killing



TEXT FIG 1*a–g* Schematic illustration of different types of lesions made on the spinal cord The striped areas indicate the extent of the lesion The ventral direction is indicated by the arrow

About 100 normal rats were studied

The animals were killed by decapitation under light ether anaesthesia. Some of the normal rats were anaesthetized with pentobarbital sodium (50 mg/kg i p Nembutal Abbott) or phenobarbital sodium (100 mg/kg i p) one to two hours before killing and in some rats (5) *in vivo* dissection of the spinal cord was performed with the animals under pentobarbital narcosis (50 mg/kg i p) Pieces from the cervical thoracic and lumbosacral part of the spinal cord the medulla oblongata pons mesencephalon and diencephalon were dissected out in all animals In the operated animals special care was taken to take out the part of the spinal cord just above and below a lesion or if spinal root lesions had been made the segments of the spinal cord from which the lesioned roots derived In all the long time and in many of the short time animals with partial transections the piece with the lesion was fixed in neutral 10% formaline dehydrated, embedded in paraffin sectioned and stained with toluidine blue to check the accuracy of the operation This however could be checked fairly well also in the freeze dried preparations The medulla oblongata pons mesencephalon and diencephalon were freeze dried treated with formaldehyde gas embedded sectioned etc as described previously (DAHLSTRÖM and FUXE 1964*a*) The spinal cord specimens required other conditions to give an optimal histochemical reaction (see below) Both cross and longitudinal sections were made After examination and photography in the fluorescence microscope a large number of sections of the medulla oblongata of normal nialamide treated and totally transected rats and rats with half side lesions at C4 (the animals having been operated on 1 4 6 12 16 19 and 40 days before killing) were after fixation stained with toluidine blue or EINARSON's chromalum gallocyanin (see DAHLSTRÖM and FUXE 1964*a*) to check for signs of chromatolysis in the CA or 5 HT nerve cells The same procedure was used to study the degenerative changes occurring in the cell bodies of the sympathetic lateral column and the anterior horn after transection of the spinal roots In this way it was possible to establish whether or not the CA or 5 HT terminals made intimate contacts with spinal nerve cells showing retrograde cell reaction

A modified technique for the histochemical demonstration of NA and 5 HT in the spinal cord

In the early stages of work on the spinal cord it was found that the fluorescence reaction varied considerably and that a good reaction was obtained only occasionally with the technique used even though this gave satis

factory results with the other parts of the central nervous system Experiments showed that the freeze dried spinal cord is unique in that for optimal conversion of the amines into their fluorescent products it seems to require a higher water content at the sites of the histochemical reaction than any of the other tissues studied in this laboratory This source of error can be avoided and reproducible results obtained with the following technique

The pieces were freeze dried in the usual manner and transferred to an exsiccator which was evacuated (ordinary water pump) Air of 90 % relative humidity was then let in slowly during ½ to 1 min The pieces were immediately taken out and transferred to a reaction vessel containing paraformaldehyde which had been equilibrated with an air atmosphere of 70–90 % relative humidity (see HAMBERGER MALMFORS and SACHS 1965) After formaldehyde treatment the pieces were embedded sectioned and mounted as previously described (DAHLSTRÖM and FUXE 1964a)

I Appearance, distribution and synaptic connections of the NA and 5 HT terminals in the spinal cord

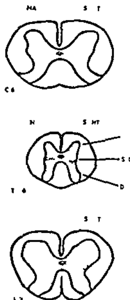
In a previous study (CARLSSON, FALCK, FUXE and HILLARP 1964) there were found a large number of fine varicose green or yellow fluorescent nerve fibres throughout the grey matter at different levels of the spinal cord There is very strong evidence that these fibres contain NA and 5 HT respectively There is little doubt that they are true synaptic terminals and that the varicosities which contain very high concentrations of the amines are presynaptic structures specialized for the synthesis storage and release of the amines This has been discussed in detail in the third paper of the present series (FUXE 1965)

No certain increase in the fluorescence intensity was observed in the 5 HT or in the NA terminals in the spinal cords dissected *in vivo* (pentobarbital anaesthesia) or taken from animals decapitated under pentobarbital or phenobarbital anaesthesia as compared with rats killed by decapitation under light ether anaesthesia

The *NA terminals* present a fairly uniform appearance throughout the grey matter and are very similar to those present in many brain nuclei (*e g* nuc hypoglossus nuc motorius n trigemini) The round to oval varicosities vary considerably in size (diameter mainly between 0 3 and 2 0 μ) and fluorescence intensity the small varicosities exhibiting a medium and the large ones a strong fluorescence intensity Most of them are below or around 1 μ in size No distinct differences in the appearance of the terminals seem to exist as between the various anatomical or functional systems The terminals showed no obvious increase in fluorescence following nialamide treatment

As previously described the NA terminals are abundantly accumulated in the sympathetic lateral column and are numerous also in the ventral horn and in the most dorsal part of the posterior horn (see Textfig 2)

The *5 HT terminals* also show a fairly uniform appearance Their distribution and appearance are best studied in reserpinized animals treated with nialamide or in animals treated with α methyl meta tyrosine (*cf* DAHL-

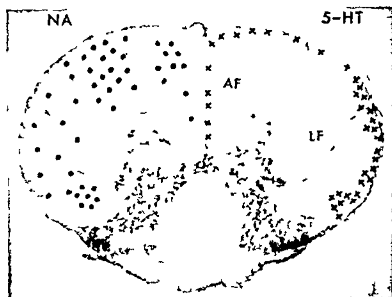


TEXT FIG 2 Schematic drawing of cross sections of the spinal cord at the levels of C6 Th6 and L2 The topography of the CA terminals are indicated with dots the 5 HT terminals with crosses VH ventral horn SLC sympathetic lateral column DH dorsal horn

STROM and FUXE 1964*a*) In such animals the 5 HT terminals are readily observed since the fluorescence of the NA terminals has disappeared Most of the 5 HT terminals in the spinal cord can be seen quite distinctly unlike those in the brain The varicosities are of about the same size as those of the NA terminals and exhibit a weak to strong fluorescence The various anatomical or functional systems do not seem to differ markedly as regards the general appearance of their 5 HT terminals —Following the administration of nialamide practically all the varicosities became strongly fluorescent and more terminals were observed than in untreated animals

The 5 HT terminals are distributed in essentially the same way as the NA terminals (see Textfig 2) but are somewhat less numerous than the latter—although densely packed—in the sympathetic lateral column and much less numerous than the NA terminals in the dorsal horn In the lower lumbar and the sacral part of the spinal cord on the other hand their number is much higher as compared with the other parts and in the ventral horn of these regions they are even more frequent than the NA terminals

The *sympathetic lateral column* which is lacking in the cervical portion of the cord and most developed in the upper thoracic portion shows a dense plexus of both types of terminals (Figs 15 16) The majority of the NA terminals seem to be concentrated in the ventromedial part (Fig 17) where they intimately enclose the cell bodies and thus to a large extent—and possibly mainly—form axo somatic synapses Most of the 5 HT terminals

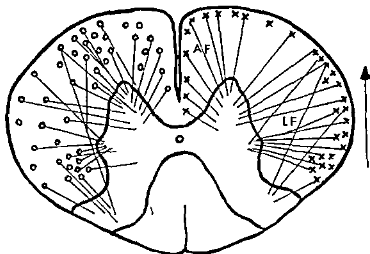


TEXT FIG 3 A microphotograph of the spinal cord at Th1 NA staining The distribution and *relative* number of descending NA and 5 HT fibres in the anterior and lateral funiculus are illustrated The total number of monoamine fibres is not in any way reflected The NA fibres are indicated with dots the 5 HT fibres with crosses AF anterior funiculu LF lateral funiculus

these types of lesions The reverse picture was obtained on the other hand if the largest part of the lateral funiculus was destroyed bilaterally (Text fig 1*e*) a very marked reduction was observed in the number of NA terminals in the sympathetic lateral column and the dorsal horn while those in the anterior horn remained essentially intact Lesions involving only the dorsolateral funiculus bilaterally gave a somewhat less marked reduction in the number of NA terminals in the sympathetic lateral column and the dorsal horn (Textfig 1*d*) Transection of the superficial zone of the dorsal horn bilaterally produced no obvious decrease in the number of NA terminals in this zone nor did lesion of the posterior funiculi change the normal fluorescence microscopical picture (Textfigs 1*f* and *g*)

There thus appear to exist two main descending systems of NA fibres in the white matter *one large system which runs in the anterior funiculus and in the most ventral part of the lateral funiculus terminating in the ventral horn and another somewhat smaller system which runs in the lateral funiculus especially in its dorsal part terminating in the sympathetic lateral column and the dorsal horn* (Textfig 4)

Lesions involving only one half of the spinal cord (Textfig 1*a*) produce approximately the same decrease in the number of NA terminals in the



TEXT FIG 4 Schematic drawing of a cross section of the spinal cord at the upper thoracic region Besides demonstrating schematically the distribution and relative number of NA and 5 HT fibres the figure roughly illustrates from which NA and 5 HT fibres the NA and 5 HT terminals in the ventral and dorsal horn and the sympathetic lateral column originate The NA fibres are indicated with circles the 5 HT fibres with crosses For abbreviations see text fig 3

sympathetic lateral column on both sides In the ventral and dorsal horn a marked to very marked reduction in the number of terminals is observed on the ipsilateral side No certain decrease is observed on the other side *About half of the NA fibres to the sympathetic lateral column appear to be crossed Crossing at spinal levels is thus much less frequent in the innervation of the ventral and the dorsal horn*

Large or small bilateral lesions of the anterior funiculus (Textfig 1*b* and *c*) both cause a fairly marked reduction in the *5 HT terminals* of the ventral horn especially in the lumbar region while the 5 HT terminals in the sympathetic lateral column are not affected Bilateral lesions affecting the greater part of the lateral funiculus give a marked to very marked reduction in the number of terminals in the sympathetic lateral column and in the dorsal horn and a fairly marked reduction in the number of terminals in the ventral horn especially in the lateral part After maximal lesions of the lateral funiculi (see Textfig 1*e*) there is obtained a very marked reduction or more often a complete disappearance of both the NA and 5 HT terminals in the sympathetic lateral column and the dorsal horn Lesions of

the dorsolateral part of the lateral funiculi (Textfig 1*d*) give a somewhat less marked reduction of the 5 HT terminals in the sympathetic lateral column and dorsal horn and an apparently normal content of 5 HT terminals in the ventral horn Lesions involving the dorsal horn and the posterior funiculi have no obvious effect

The 5 HT terminals in the ventral horn are thus derived mainly from 5 HT fibres in the medial part of the anterior funiculus and from fibres in the anterior part of the lateral funiculus The 5 HT terminals in the sympathetic lateral column and dorsal horn derive from fibre tracts in the lateral funiculus present especially in its dorsal part (Textfig 4) *The 5 HT terminals show the same type of crossing pattern at spinal levels as the NA terminals*

It is possible however that some of the NA fibres in the white matter do not terminate in the grey matter of the spinal cord but leave the spinal cord via the anterior spinal roots to terminate somewhere outside the central nervous system It has been found that on the central side of a transection of the anterior roots CA rapidly accumulates in nerve fibres which can sometimes be traced from the white matter of the spinal cord (DAHLSTRÖM and FUXE 1965)

3 ACCUMULATION OF NA AND 5 HT IN DESCENDING FIBRES ABOVE A TRANSECTION OF THE CORD

Following axotomy such large amounts of amines rapidly accumulate in the proximal part of central monoamine nerve fibres (Fig 21) that the presence distribution and direction of the latter can be easily studied (Figs 22 23) (DAHLSTRÖM and FUXE 1964*b*) This method has been used *e g* to map out the nigro neostriatal DA neuron system (ANDÉN CARLSSON DAHLSTRÖM FUXE HILLARP and LARSSON 1964 ANDÉN DAHLSTRÖM FUXE and LARSSON 1965*a*) It has been shown that the amines accumulating in the transected fibres are the monoamines normally formed and stored by the respective neurons (DAHLSTRÖM 1965 DAHLSTRÖM and HÄGGENDAL unpublished results ANDÉN HÄGGENDAL and MAGNUSSON unpublished data)

Preliminary data on the accumulation of NA and 5 HT in transected descending fibres of the spinal cord (DAHLSTRÖM and FUXE 1964*b*) have been confirmed in the present paper A detailed description of this will be given in a later paper

Following a transection of the spinal cord an accumulation of NA and 5 HT could be observed already after 7 h reached a peak after about 24 h and then remained fairly unchanged up until the 12th day The fibres were swollen and distorted and showed an amine accumulation up to approxi

mately 2 mm above the transection (Figs 21 22) Later still the amines gradually di appeared and after 40 days no accumulation could be observed

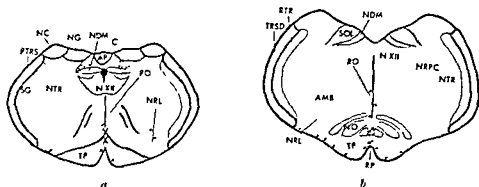
If animals were treated with nialamide after the spinal transection it could be directly observed how the smooth thin 5 HT fibres descending in the white matter became swollen and distorted exhibiting an intense fluorescence in the zone above the lesion Thus the accumulated amines—at *least the 5 HT—are present within true monoamine fibres* *Following* the administration of nialamide the distal part of the transected fibres also showed markedly increased 5 HT levels up to 5 days after operation giving evidence for the occurrence of amine synthesis in these fibres

The distribution pattern of the descending NA and 5 HT fibres at the level of C8 is given schematically in Textfig 3 No differences were observed between the distribution of fibres showing an accumulation of 5 HT due to transection or to nialamide treatment (Section II 1) Large numbers of NA fibres were present in the anterior funiculus (Fig 24) especially in the border zone between the anterior and lateral funiculi where many fibres *of weak to medium green fluorescence were observed al o in normal animals* (Section II 1) Numerous NA fibres were found also in the lateral funiculus but were more scattered than the 5 HT fibres and localized deeper in the white matter (Fig 25) No descending monoamine fibres at all could be detected in the dorsal superficial zone of the posterior horn or in the posterior funiculus In *cat* the distribution of the NA and 5 HT fibres as observed after axotomy was essentially the same as in rat However some differences were observed The 5-HT fibres were much more frequent in the superficial medial part of the anterior funiculus and much less frequent in the superficial ventral part of the lateral funiculus in cat than in rat Furthermore the NA fibres of cat were not as frequent in the anterior funiculus as those of rat

Amine accumulation however was observed not only in descending fibres but al o in certain of the 5 HT and NA terminals of the ventral horn and sympathetic lateral column just above the lesion These terminals showed increased fluorescence intensity and their varicosities were often swollen

III Localization of the cell bodies of the bulbo spinal NA and 5 HT neuron systems

The cell bodies of the specific monoamine neurons in the brain normally show very low to low levels of their respective amines but these can be very markedly increa ed either by MAO inhibition (5 HT neurons) or by



TEXT FIG 5a–b Schematic drawing of transverse sections of the medulla oblongata at the level of the area postrema (a) and of the anterior third of the inferior olivary complex (b) The topography of groups B1–B2 and A1–A2 is schematically illustrated The CA cell bodies are indicated with dots the 5 HT cell bodies with crosses AMB nuc ambiguus AP area postrema C nuc commissuralis N XII nuc hypoglossus NC nuc cuneatus NDM nuc motorius dorsalis n vagi NG nuc gracilis NO olivary complex NRL nuc reticularis lateralis NRPC nuc reticularis parvocellularis NTR nuc tractus spinalis n trigemini RO nuc raphe obscurus RP nuc raphe pallidus RTRS radix tractus spinalis n trigemini SG substantia gelatinosa SOL nuc tracti solitarii TP tractus pyramidalis TRSD tractus spino cerebellaris dorsalis

the administration of L-DOPA (CA neurons) to animals pretreated with nialamide (DAHLSTRÖM and FUXE 1964a FUXE 1965 FUXE and HILLARP 1965) Neither in normal animals nor in animals treated in these ways have nerve cells exhibiting specific fluorescence been detected in any part of the spinal cord No such cells were found following transection of the cord at different levels There is thus little doubt that the descending NA and 5 HT fibres originate from supraspinal centres

Both CA and 5 HT nerve cells have been found in the medulla oblongata (see DAHLSTRÖM and FUXE 1964a) The largest CA cell groups have been termed A1 and A2 and the 5 HT cell groups B1 B2 and B3 (see DAHLSTRÖM and FUXE 1964a and Textfig 5) Group A1 is localized in the ventro lateral part of the reticular formation in the caudal part of the medulla oblongata and A2 mainly within the nuc commissuralis Groups B1 and B2 are localized mainly to the nuc raphe pallidus and obscurus respectively B3 is a large group which surrounds mainly the pyramidal tracts at different levels of the facial nucleus but is also present within the nuc raphe magnus The cells of groups A1 and A2 usually show a weak to medium fluorescence intensity while that of the 5 HT groups is very weak to weak After nialamide treatment however they show even with a low dose (50 mg/kg) a medium to strong fluorescence intensity while the CA cells have about the same intensity as in normal animals

After nialamide treatment it was possible in horizontal and sagittal sections to see how most of the cell bodies of groups B1 and B2 sent yellow fluorescent axons down into the spinal cord The axons run first in a ventrolateral direction This caused them to approach the ventral surface of the brain fairly quickly and in the borderzone towards the spinal cord they could be seen to form a narrow band under the part of the ventral surface that was localized lateral to the pyramidal tract (Fig 18) CA axons were observed also in the borderzone towards the spinal cord lying laterally of the ventral horn of C1 They could not however be traced back to any CA cell bodies

Following a total transection at C8 a large number of A1 cells (Fig 26)—especially in the rostral part of the group—and some A2 (Fig 28) cells showed a very marked increase in fluorescence intensity 2 to 4 days after operation No certain increase was observed after 7 h but a fairly large increase in fluorescence intensity had occurred after one day During the period 4 to 12 days after transection the very strong fluorescence intensity gradually decreased to the weak or medium intensity seen in normal animals This fluorescence did not show any further decrease in intensity not even after 40 days In some A1 cells however a strong fluorescence intensity could still be observed 5 to 6 days after transection No increase in fluorescence intensity was observed in the CA cells in the pons or mesencephalon nor was any certain increase in fluorescence intensity observed in the 5 HT nerve cells in the medulla oblongata or the rest of the lower brain stem

Practically all of the nerve cell bodies with an increased fluorescence intensity appeared swollen when compared with cell bodies of normal intensity (Figs 27*a–b*) This change was observed more or less parallel with the increase in fluorescence intensity Also most of the cells with increased fluorescence had an eccentrically situated nucleus

If the transections were made at Th 12 instead of C8 the number of A1 and A2 cells with an increased fluorescence intensity was considerably reduced If total transection at C8 were made in young rats (4 weeks old) the number of A1 and A2 cells showing increased intensity was considerably increased the majority of the A1 cells being affected

No clear chromatolysis was observed in the *A1* and *A2* cells nor in the B1 B2 and B3 cells during the time period studied after total and half side transections The failure to show any chromatolysis is probably due to the well known fact that it is very difficult to detect any chromatolysis in small nerve cells with a low amount of Nissl substance

After a large bilateral lesion of the anterior funiculus involving also the

most ventral part of the lateral funiculus (Textfig 1c) but not after a small one (Textfig 1b) a fairly large number of A1 cells showed increased fluorescence intensity although the number was about 40% lower than in animals with a total transection Following the bilateral lesion of the lateral funiculus alone the number of A1 cells with increased fluorescence intensity was reduced to about 40% of the normal value If only the dorsal parts of the lateral funiculus (Textfig 1d) were damaged bilaterally the A1 cells with increased fluorescence were less in number than after a transection of the entire lateral funiculus bilaterally (Textfig 1e) No A1 cells with increased fluorescence intensity were observed after lesions of the posterior horns or the posterior funiculi In the animals with a lesion of only half of the spinal cord the number of A1 cells showing increased fluorescence intensity was about equal on the two sides the total number of such cells being about 50% of that in animals with total transections Assuming that the increased CA levels in the A1 cell bodies are a reaction to axotomy (see Discussion) the results from the partial transections suggest that most of the A1 cells proceeding to the spinal cord send axons to the most ventral part of the lateral funiculus and the anterior funiculus while a somewhat minor proportion seem to send axons to the dorsal part of the lateral funiculus

Nialamide treatment (500 mg/kg) produced no change in the appearance of the CA cells of the A1 and A2 groups After different types of lesions the 5 HT cells of groups B1 to B3 (Fig 31) showed the same very marked increase in fluorescence intensity (Figs 29 30 32) following treatment irrespective of what lesions that had been made and how many days after operation the animals were killed With a low dose of nialamide (50 mg/kg) however it was found in animals killed 8 to 12 days after a total transection at C8 that some of the B1 B2 and above all the B3 cells did not show the same increase in fluorescence intensity as in normal animals but a slightly lesser one This could be due to a decrease in the capacity of the nerve cell bodies to form 5 HT No clear chromatolysis was observed in these cells

Reserpine treatment (4 h) in animals which had been totally transected for 2 days resulted in the appearance of A1 cells with at most a weak to medium fluorescence intensity In other words a large decrease in fluorescence intensity had occurred in the cytoplasm of the A1 cells that after total transection developed a very strong fluorescence intensity 24 h after the reserpine treatment however the swollen cell bodies were found to have the same high fluorescence intensity as the same cells in animals with a total transection only

DISCUSSION

In a previous paper (CARLSSON FALCK FUXE and HILLARP 1964) strong support was obtained for the view that the NA and 5 HT terminals in the grey matter of the spinal cord belong to descending systems of supraspinal NA and 5 HT neurons There is little doubt that these terminals represent terminal axon ramifications and that they are true synaptic terminals (CARLSSON FALCK FUXE and HILLARP 1964 FUXE 1965) In the present paper strong evidence is given for the existence of several large bulbospinal NA and 5 HT neuron systems

The evidence is as follows

1 The majority of 5 HT nerve cells in the caudal ventromedial part of the medulla oblongata (Groups B1 to B3) can after nialamide treatment be seen to send axons down into the spinal cord 5 HT terminals are seen to arise from these fibres terminating in the ventral horn or the sympathetic lateral column The entire 5 HT neuron can thus be directly visualized in the microscope Furthermore total transection is in many of these cells followed within 8 to 12 days by degenerative changes in the form of a somewhat decreased capacity to accumulate 5 HT after MAO inhibition

2 CA nerve cells (mainly group A1) are found in the caudal part of the medulla oblongata After total transection of the spinal cord these cell bodies show a very marked increase in their amine contents in all probability due to axotomy (see below) Furthermore CA fibres can be traced from the white matter of the spinal cord up to the most caudal part of the medulla oblongata and no further

3 Practically all monoamine terminals disappear below the lesion 6 to 8 days after a total transection (see also CARLSSON FALCK FUXE and HILLARP 1964) This disappearance runs parallel with an almost complete depletion of the monoamine stores as determined biochemically (ANDEN HAGGENDAL MAGNUSSON and ROSENGREN 1964) Furthermore the high capacity of the 5 HT terminals and non terminal axons present below the lesion to form and accumulate 5 HT after MAO inhibition is observed only during the first 5 days after operation This suggests that in addition to the inability to store 5 HT the synthesis of 5 HT has ceased This is in good agreement with the marked decrease in DOPA decarboxylase activity oc

curring in the part of the spinal cord below the lesion during the first 10 days after operation (ANDÉN, MAGNUSSON and ROSENGREN 1965) This enzyme is known to take part in the synthesis of both CA and 5 HT

4 There is a marked and rapid accumulation of NA and 5 HT in mono amine fibres just above—but not below—a lesion in the spinal cord at the level studied This is in all probability due to a transport of amine storage granules from the cell bodies to the terminals (DAHLSTRÖM and FUXE 1964b DAHLSTRÖM 1965, DAHLSTRÖM FUXE and HILLARP 1965)

There is strong evidence that the central monoamine neurons are mono aminergic *i e* function by releasing their amines from the synaptic terminals (DAHLSTRÖM and FUXE 1964a, FUXE 1965) Recent investigations on the spinal cord have furnished further evidence for this view *In vitro* stimulation of the spinal cord has resulted in a release of 5 HT and NA from the cord into the incubation bath and in an increased synthesis of 5 HT and NA (ANDÉN CARLSSON, HILLARP and MAGNUSSON 1964 1965) Furthermore, electrical stimulation of the medulla oblongata *in vivo* in conjunction with synthesis inhibition causes a marked depletion of the NA and 5 HT stores in the varicosities of the terminals (DAHLSTRÖM FUXE KERNELL and SEDVALL 1965) There is thus little doubt that NA and 5 HT are released from the terminals and thus may act as transmitters in these neuron systems This view is supported by neurophysiological studies (ANDÉN LUNDBERG ROSENGREN and VYKLICKY 1963 ANDÉN JUKES and LUNDBERG 1964 ANDÉN JUKES LUNDBERG and VYKLICKY 1964) and studies on inhibition by 5 HTP of the insulin induced adrenaline depletion of the adrenal medulla (ANDÉN CARLSSON and HILLARP 1964)

The highest concentration of 5 HT (1 4 μg/g) has been found in the conus medullaris of cat This concentration is 5 times higher than in the thoracic cord (ANDÉN 1965) Histochemically the sacral region is found to be much richer in 5 HT terminals than the other parts of the spinal cord The 5 HT terminals show no pronounced accumulation to particular nuclei but lie on the whole fairly scattered Biochemical determinations of the amine contents of the ventral horn lateral horn and dorsal horn in cow (ANDÉN 1965) agree very well with the distribution of the monoamine terminals in the mammals studied in this investigation High concentrations of NA and 5 HT were found in the lateral horn where there is a very dense accumulation of NA and 5 HT terminals From the quantitative point of view however these terminals constitute only a minor proportion of the terminals in the spinal cord The great majority are to be found in the dorsal and especially in the ventral horn

In the dorsal horn the CA terminals are concentrated to the apex, which

is mainly occupied by the substantia gelatinosa The peculiar parallel arrangement of the CA terminals observed in this region has been found also in the retrochiasmatic area of the hypothalamus but in no other region The terminals in the substantia gelatinosa probably make intimate contacts with other fibres However they probably mainly form parallel contacts with the abundant dendrites in this region which also have a cranio caudal orientation (see CAJAL 1899)

In the ventral horn the monoamine terminals are present mainly in the areas containing the cell bodies of the α motoneurons to the skeletal musculature (REXED 1954) The area containing the medial motor cell groups innervating the axial musculature receives large numbers of 5 HT and NA terminals in the thoracic part of the spinal cord but much fewer in the intumescences This suggests a more efficient supraspinal control over the part of the axial musculature involved in respiration Parts of the area of the lateral motor cell bodies which innervate the muscles of the extremities contain a large number of monoamine terminals These parts seem to correspond mainly to the ventrolateral and above all to the dorsolateral cell groups The area which seems to correspond to the retro dorsolateral cell group contains on the other hand much fewer terminals indicating differences in the supraspinal control as between these different cell groups Since *e g* the flexor and extensor muscles of the forearm and hand and of the leg and foot receive innervation from the dorsolateral group while the retro dorsolateral group supplies the small muscles of the foot and hand it is probable that the former muscles are influenced more than the latter by the activity of the monoamine neurons (see CROSBY HUMPHREY and LAUER 1962)

The fact that the NA and 5 HT terminals make intimate contacts directly with the α motoneurons and the autonomic nerve cells in the sympathetic lateral column strongly suggests the existence of monosynaptic pathways directly from the reticular formation and raphe region of the medulla oblongata to these cells It must be remembered however that the varicosities may form synaptic junctions not with the cell bodies but with other terminals which in turn form axo somatic junctions Electron microscopic observations of the motor cell bodies of the trigeminal nerve which like the motoneurons of the spinal cord are intimately surrounded by NA and 5 HT terminals reveal however that at least the vast majority of the structure form axo somatic junctions Axo axonic junctions are very seldom observed (FUXE and HOKFELT unpublished data)

The marked increase observed in the amine contents of CA cells (especially group A1) after transection of the spinal cord may be due either to a

change in impulse flow to these cells or to retrograde changes in rate of synthesis caused by axotomy. Many findings give strong support to the latter alternative

1. The nerve cell bodies with *increased amine contents are often swollen* and have an eccentric nucleus. These phenomena are characteristic of the retrograde cell reaction

2. The nerve cells show a maximum amine content between 2 and 4 days, followed by a gradual decrease in intensity to the normal. The retrograde changes observed have the same time course and the cells appear normal after 5 to 10 days

3. More cells with increased CA contents are observed in young than in adult animals. It is known that retrograde cell changes are much more marked in young than in adult animals

The increased CA contents in the cells (above all group A1) is thus in all probability due to retrograde changes following axotomy. Consequently there is little doubt that these cells belong to NA reticulo spinal neurons giving rise to NA terminals in the spinal cord. A similar although weaker increase in amine content has been observed in the nerve cells (group A9) belonging to the large nigro neostriatal DA neuron system after removal of the neostriatum (ANDÉN, CARLSSON, DAHLSTRÖM, FUXE, HILLARP and LARSSON 1964; ANDÉN, DAHLSTRÖM, FUXE and LARSSON 1965*a*). The weaker increase obtained may be due to the fact that only the terminals were removed. Furthermore a fairly strong increase in the amine contents of the CA cells of group A10 has been found after lesion of the medial fore brain bundle (ANDÉN, DAHLSTRÖM, FUXE and LARSSON 1965*b*). *The accumulation of amines in the proximal neuron parts after axotomy has thus proved a useful method of mapping out the CA neuron systems.* The CA cell bodies seem to show differences in sensitivity to axotomy, since after 4 weeks the cells of group A1 still have a normal fluorescence intensity, while many cell bodies of group A9 show a marked decrease in fluorescence intensity together with degenerative changes (ANDÉN, DAHLSTRÖM, FUXE and LARSSON 1965*a*)

It is impossible at the present time to explain why the amine contents of the NA cell bodies increase after axotomy. It may be that axotomy induces an increased formation of amine granules or a decreased transport of the granules from the cell bodies. These explanations are to a certain extent opposed by the fact that the nerve cells belonging to the bulbospinal 5 HT neurons do not increase their contents of 5 HT following axotomy

The origin and course of the reticulo spinal NA neurons demonstrated in this paper are to a large extent identical with those reticulo spinal fibres

in cat (TORVIK and BRODAL 1957) which are known to arise from the nuc reticularis lateralis and after partial crossing descend in the border zone between the lateral and anterior funiculus A large proportion of the reti culo spinal NA fibre systems can thus in all probability be identified with already known fibre systems These authors however found no projection of reticulo spinal fibres to the lumbar and sacral segments Later investi gations on the other hand indicate that *most* reticulo spinal neurons descend to lumbar segments (WOLSTENCROFT 1964) However these neurons are in all probability not identical with the 5 HT and NA neurons project ing to the lumbar segments since the axons described by this latter author have too high conduction velocities to be identical with the thin mono amine axons

The discovery of raphe spinal 5 HT systems with cell bodies to a large extent in the caudal raphe nuclei is in agreement with the results of BRODAL TABER and WALBERG (1960) who found that the nuc raphe obscurus nuc raphe pallidus and above all nuc raphe magnus send fibres to the spinal cord

Little is known as to the function of the descending monoaminergic neurons to the spinal cord There exist however indications that 5 HT terminals in the sympathetic lateral column may have an inhibitory func tion (ANDEN CARLSSON and HILLARP 1964) In a previous paper (CARLS SON FALCK FUXE and HILLARP 1964) it was suggested that 5 HT terminals in the sympathetic lateral column have also a vasodepressor function since the cell bodies of the 5 HT neurons to a very large extent are localized in the vasodepressor area and the descending 5 HT fibres to a large extent have the same localization as the fibres from this area (LIM WANG and YI 1938 ALEXANDER 1946) It should be pointed out however that the view introduced by these authors of a well defined vasodepressor centre giving fibres mainly to the dorsolateral funiculus must be re evaluated Later studies (GUTMAN GINATH CHAIMOWITZ and BERGMANN 1962 GUTMAN LEI BOWITZ and BERGMANN 1962) support the view that no anatomically well defined antagonistic vasomotor centres in fact exist and it has been shown that autonomic fibres such as the vasopressor fibres descend in very super ficial parts of the *entire* lateral funiculus (KERR and ALEXANDER 1964) In *view of these findings the suggestion that the 5 HT neurons terminating* in the sympathetic lateral column may be vasodepres ors must be treated with great caution There is little doubt however that a true bulbo spinal inhibitory path projecting on a spinal vasoconstrictor reflex does exist (PROUT COOTE and DOWNMAN 1964)

Little can be said again of the function of the NA terminals in the sym

pathetic lateral column One finding however is noteworthy, namely that these terminals belong to NA neurons which in all probability have their cell bodies lying in a cranio caudal band in the ventro lateral part of the medulla oblongata which to a large extent seems to be identical with the vasodilator band in this area (LINDGREN and UVNÄS 1953)

As regards the function of the 5 HT and NA terminals in the ventral and dorsal horn only a few suggestions can be made By electrical stimulation in the caudal medial area of the medulla oblongata it has been possible to excite motoneurons in the spinal cord an effect that can be produced in a spinal animal also by the injection of 5 HTP which probably acts via the formation of 5 HT in monoaminergic terminals (ANDÉN JUKES and LUNDBERG 1964) Electrical stimulation is without effect after reserpinization Since the present investigation has shown that the cell bodies of the bulbo spinal 5 HT neurons are present in the area stimulated and that 5 HT terminals make intimate contacts with α motoneurons the findings taken together suggest the presence of a monosynaptic 5 hydroxy tryptaminergic excitatory bulbo spinal pathway

The injection of L DOPA which acts mainly via the formation of NA in monoaminergic terminals has been shown to suppress transmission from flexor reflex afferents to primary afferents motoneurons and ascending pathways (ANDEN LUNDBERG ROSENGREN and VYKLICKY 1963) The injection of 5 HTP has been found to have the same effects (ANDEN JUKES and LUNDBERG 1964) The effects of NA and 5 HT on the spinal reflexes from the flexor reflex afferents are not due to any direct action on the primary afferents or the motoneurons and are thus exerted at an interneuronal level The precise localization of these interneurons is not however known with certainty These results strongly underline the importance of finding out with certainty whether or not certain interneurons—and if so which—receive monoaminergic synaptic terminals In this connection it can be mentioned that the large numbers of NA terminals—and in cat also 5 HT terminals—in the substantia gelatinosa probably mainly make close parallel contacts with the abundant dendrites belonging to interneurons present in the substantia gelatinosa Since true postsynaptic inhibition has recently been evoked from the medial lower reticular formation in flexor and extensor motoneurons (JANKOWSKA LUND LUNDBERG and POMPEIANO 1964) it may be that at least some of the monoamine terminals making intimate contacts with the motoneurons have this action

Finally it cannot be excluded that a small number of monoamine neurons not belonging to any of the categories described above may give rise to terminals in the spinal cord and that some of the monoamine terminals in

the spinal cord are too fine to be visualized with the present histochemical technique it can also be stated that monoamine bulbo spinal neurons in all probability play an important role in the supraspinal control of both autonomic and somatic functions in the spinal cord The present demonstration and mapping out of various bulbo spinal NA and 5 HT neuron systems should be of considerable assistance in forthcoming experiments to elucidate the functional organization of these neuron systems

SUMMARY

With the help of the highly specific and sensitive fluorescence method of FALCK and HILLARP combined with transection experiments and/or MAO inhibition it has been possible to show that the NA and 5 HT terminals in the spinal cord in rat belong at least mainly to several descending bulbo spinal NA and 5 HT neuron systems A modified technique for the histo chemical demonstration of the amines in the spinal cord has been worked out

The NA terminals in the ventral horn derive at least mainly from fibres in the anterior funiculus and the most ventral part of the lateral funiculus The NA terminals in the sympathetic lateral column and the dorsal horn on the other hand derive from tracts in the lateral funiculus present espe cially in its dorsal part

The 5 HT terminals in the ventral horn derive at least mainly from fibres in the medial part of the anterior funiculus and the superficial ventral part of the lateral funiculus Those in the sympathetic lateral column and the dorsal horn derive from fibres in the lateral funiculus mainly in the dorsal part Both the NA and 5 HT tracts to the sympathetic lateral column un like those to the dorsal and ventral horn show a high degree of crossing at spinal levels

Most of the descending NA fibres are present in the anterior funiculus and the most ventral part of the lateral funiculus while most of the 5 HT fibres descend in the superficial part of the lateral funiculus

With the help of retrograde cell changes occurring after spinal root tran sections it has been shown that both NA and 5 HT terminals make inti mate contacts with *inter alia* cell bodies of the sympathetic lateral column and the α motoneurons In the ventral horn at the levels of the intumes cences the NA and 5 HT terminals are most frequent in the ventrolateral part in an area which seems to correspond mainly to that of the ventro lateral and dorsolateral group of the lateral motor cell bodies the latter group innervating the flexors and extensors of the forearm hand and leg foot

The NA terminals in the substantia gelatinosa have a unique appearance They descend or ascend in this zone parallel to each other without branching They probably form close parallel contacts with the abundant dendrites of

the interneurons in the substantia gelatinosa These dendrites have the same longitudinal orientation as the terminals

The nerve cells of the NA bulbo spinal neuron systems are in all probability localized mainly in the ventrolateral part of the reticular formation of the medulla oblongata (Group A1) Those of the 5 HT neuron systems are localized in the three raphe nuclei of the medulla oblongata and the part of the reticular formation immediately surrounding the pyramidal tract at these levels (Group B1 to B3) After axotomy the CA cell bodies show a very marked increase in their amine contents *This phenomenon forms the basis for a method of mapping out the CA neuron systems*

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IV

The Distribution of Monoamine Terminals in the Central Nervous System

by

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INTRODUCTION

Evidence for the existence of central monoamine neurons has been presented in earlier papers (DAHLSTRÖM and FUXE 1964a, 1965). The characteristics of the monoamine nerve terminal and its reaction to drugs interfering with monoamine metabolism have also been described (FUXE 1965). So far, however, the distribution of the central monoamine terminals has been described only in certain regions (CARLSSON, FALCK and HILLARP 1962; CARLSSON, FALCK, FUXE and HILLARP 1964; DAHLSTRÖM and FUXE 1964b, 1965; FUXE 1964, 1965). The chief aim of the present paper is to give a comprehensive report of the distribution of these terminals in the central nervous system and a more detailed description of the reaction of the various terminal systems to drugs interfering with monoamine metabolism.

Abbreviations: CA = catecholamine (s), DA = dopamine, NA = noradrenaline, A = adrenaline, 5-HT = 5-hydroxytryptamine, 5-HTP = 5-hydroxytryptophan, DOPA = 3,4-dihydroxyphenylalanine, MAO = monoamine oxidase, α-MMT = α-methyl-meta-tyrosine

MATERIAL AND METHODS

About 540 male Sprague Dawley rats (200 to 250 g 2 months old) have been used, of which about 440 were treated with drugs in accordance with Table II

The substances were dissolved and injected as previously described (DAHLSTRÖM and FUXE 1964a) The brain and the spinal cord were divided as described by FUXE (1965) and DAHLSTRÖM and FUXE (1965) respectively The part of the forebrain cranial to the neostriatum was also studied however The pieces were freeze-dried treated with formaldehyde gas embedded in paraffin sectioned mounted and examined as previously described (DAHLSTRÖM and FUXE 1964a)

RESULTS

I Localization of the monoamine nerve terminals

The distribution of the CA and 5 HT terminals is summarized in Table I The word density in Table II is used to denote the number of monoamine terminals per square of section surface with a 100 μ side Five different degrees are used to evaluate the density in any given area namely very low (1+) low (2+) medium (3+) high (4+) and very high (5+) A very high density is illustrated in parts of Figs 16 17 33 36 37 43 53 59 60 a high density in Figs 11 13 14 34 62 a medium density in Figs 51 54 55 61 and Figs 9 and 10 in Fuxe 1965 a low density in Figs 11 12 56 57 a very low density in Figs 52 61 64 Depending on the diameter of the varicosities of the majority of the terminals in a given nucleus or area the terminals will be described as very fine (mainly around 0 5 μ thick) fine (mainly around 1 μ thick) fairly thick (mainly between 1 0 and 2 5 μ thick) or thick (mainly 2 5 to 4 μ thick) In some areas a finely *dotted* or *diffuse* specific fluorescence is observed instead of typical terminals examples being respectively the dorso lateral part of the nuc interstitialis striae terminalis and the nuc caudatus putamen This is due to the presence of very fine partly sub lightmicroscopical terminals which are so closely packed that the individual terminal can only be observed under perfect conditions (see Fuxe 1965) Such accumulations of terminals will be referred to in the Table as *dotted* and *diffuse* respectively

There exist two types of CA terminals namely DA and NA terminals There is little doubt that most of the terminals in the neostriatum the tuberculum olfactorium and the external layer of the median eminence store DA and so very probably do the CA terminals in the nuc accumbens and certain circumscribed areas just medial to it partly within the nuc tractus diagonalis (Fuxe 1965 Carlsson Dahlström Fuxe and Hillarp 1965) On the basis of α MMT administration which—if used with caution—can be employed as a tool for differentiation between NA and DA it seems probable that some of the CA terminals in certain other areas of the brain store DA (*cf* Fuxe 1965 and Table I)

The 5 HT terminals are best studied after a high dose of nialamide especially in previously reserpine treated animals since on MAO inhibition a

TABLE 1 *The distribution of monoamine terminals in the central nervous system of the rat*

	Density of CA terminals[a]	Density of 5 HT terminals[b]
Spinal cord		
I *Posterior horn at all levels*		
1 The most superficial parts (correspond to the substantia gelatinosa) (Figs 11 12 13 and 14)	4+ fine (NA)	scattered fine
2 The other parts of the dorsal horn (Figs 11 12)	2+ fine (NA)	1+ fine
II *Anterior horn*		
A Cervical and lumbar enlargement		
1 Lateral motor group		
a) ventrolateral and dorsolateral groups (Figs 1 2 3)	3+ to 4+ fine (NA)	3+ to 4+ fine
b) retrodorsolateral group (Figs 1 2)	2+ fine (NA)	2+ fine
2 Medial motor group	2+ fine (NA)	2+ fine
B Thoracic part (Fig 4)		
1 Medial motor group (Figs 5–10)	3+ to 4+ fine (NA)	3+ fine
III *Lateral horn*		
Sympathetic lateral column (Figs 15 16 17)	5+ fine	5+ fine
(? The sacral autonomic nucleus in cat)	1+ fine	3+ fine
Lower brain stem		
I *The raphe nuclei* (see comments to Table 1)		
1 Nuc raphe pallidus (Figs 7 and 10 in DAHLSTRÖM and FUXE 1964a Fig 4 in FUXE 1965)	5+ fairly thick (mainly NA probably some DA)	—
2 Nuc raphe obscurus	4+ fairly thick (mainly NA probably some DA)	—
3 Nuc raphe magnus	3+ fairly thick (mainly NA probably some DA)	scattered very fine
4 Nuc raphe pontis	1+ fine (NA)	—
5 Nuc raphe dorsalis	2+ to 3+ fine (NA)	—
6 The rest of the raphe complex in the mesencephalon	1+ fine (NA)	—

[a] Normal animals

[b] Animals treated with a large dose of a potent MAO inhibitor — such as nialamide

	Density of CA terminals	Density of 5 HT terminals
II *The reticular formation encompasses the reticular nuclei described by* VALVERDE (1962) (see comments to Table 1 Figs 44 54 55 56 57 Figs 6 7 15 in FUXE 1965)	scattered to 4+ very fine to fine (NA)	scattered to 2+ very fine to fine
III *Cranial nerve nuclei*		
A Somatic efferent nuclei		
1 Nuc n oculomotorii	—	1+ fine
2 Nuc n trochlearis	—	1+ fine
3 Nuc n abducens	scattered fine (NA)	2+ fine
4 Nuc prehypoglossus	3+ fine (NA)	3+ fine
5 *Nuc hypoglossus (Fig 63 Figs* 9 and 10 in FUXE 1965)	3+ to 4+ fine (NA)	3+ fine
B Special visceral efferent nuclei		
1 Nuc motorius n trigemini (Fig 62)	4+ fine (NA)	4+ fine
2 Nuc n facialis	4+ fine (NA)	4+ *fine*
3 Nuc ambiguus	2+ fine (NA)	2+ fine
C General visceral efferent nuclei		
1 Nuc Edinger Westphal pars rostralis	1+ fine (NA)	—
2 Nuc Edinger Westphal pars caudalis (GILLILIAN 1943)	4+ fine (NA)	—
3 Nuc salivatorius superior (SHUTE and LEWIS 1960)	—	3+ fine
4 Nuc motorius dorsalis n vagi (Figs 42 43 59 Figs 1 3 and 17 *in* FUXE 1965)	5+ fine to fairly thick (mainly NA probably *some DA)*	4+ fine
D Visceral afferent nuclei		
1 Nuc tracti solitarii (Fig 42)	4+ to 5+ fine to fairly thick (mainly NA probably some DA)	1+ to 3+ fine
2 Nuc commissuralis (Fig 43 Fig 3 in FUXE 1965)	4 to 5+ fine (mainly NA probably some DA)	1+ to 3+ *fine*
3 Nuc intercalatus	1+ fine (NA)	3+ fine
4 Nuc parasolitarius	2+ to 3+ fine (NA)	1+ fine
E General somatic afferent nuclei		
1 Nuc tr mesencephalici n trigemini (Fig 19 in DAHLSTRÖM and FUXE 1964a)	2+ to 3+ fine (NA)	—
2 The rest of the sensory nuclei of n trigeminus	1+ very fine to fine (NA)	scattered

Table 1 (*cont*)

	Density of CA terminals	Density of 5 HT terminals
F Special somatic afferent nuclei		
1 Nuc cochlearis ventralis	2+ very fine (NA)	—
2 Nuc cochlearis dorsalis	scattered to 1+ very fine (NA)	scattered fine
3 All vestibulary nuclei	—	
III *Other areas in the lower brain stem*		
A Medulla oblongata		
1 Substantia gelatinosa (Figs 64 65)	2+ fine (NA)	3+ very fine
2 Nuc gracilis	1+ very fine (NA)	1+ very fine
3 a) Nuc cuneatus medialis	1+ very fine (NA)	1+ very fine
b) Nuc cuneatus externus	scattered very fine (NA)	—
4 Inferior olivary complex		
a) Nuc olivaris inferior principalis (dorsal part) (Figs 2 20 in FUXE 1965)	5+ very fine (NA)	—
b) Nuc olivaris inferior principalis (ventral part) (Figs 2 20 in FUXE 1965)	2+ fine (NA)	scattered very fine
c) Nuc olivaris accessorius dorsalis (medial part) (Fig 2 in FUXE 1965)	1+ fine (NA)	scattered very fine
d) Nuc olivaris accessorius dorsalis (caudal lateral part)	scattered fine (NA)	dotted fluorescence of strong intensity
e) Nuc olivaris accessorius medialis (Figs 2 and 20 in FUXE 1965)	3+ to 4+ fairly thick (NA)	scattered very fine
B Other areas within the Pons		
1 Superior olivary complex	—	—
2 Nuc corporis trapezoidei	—	—
3 Nuc pontis (See comments to Table 1)	1+ to 3+ very fine (NA)	—
4 Substantia grisea periventricularis (see comments to Table 1 Fig 46)	1+ to 4+ fine (NA)	1+ to 4+ very fine
C Other areas within the mesencephalon Limbic midbrain area		
1 Nuc tegmenti dorsalis (mouse)	—	3+ very fine
2 Nuc tegmenti ventralis	3+ fine (NA)	—
3 The ventromedial part of the substantia grisea centralis (Fig 53)	3+ fine (NA)	—
4 The ventrolateral part of the substantia grisea centralis (see Fig 53)	5+ fine (mainly NA probably some DA)	—

	Density of CA terminals	Density of 5 HT terminals
5 Nuc medianus raphe	scattered to 1+ fine (NA)	—
6 Area ventralis tegmenti	scattered fine (NA)	—
Areas involved in the extrapyramidal system		
1 Nuc ruber all parts	—	—
2 Substantia nigra zona compacta	scattered to 1+ fine (NA)	—
3 Substantia nigra zona reticulata	—	diffuse fluorescence of medium intensity
4 Substantia nigra pars lateralis	1+ fine (NA)	—
Remaining areas in the tegmentum		
1 Nuc interpeduncularis	2+ very fine (NA)	scattered very fine dotted fluorescence of strong intensity
2 The area just dorsal to the caudal half of the nuc interpeduncularis and ventral to the nuc medianus raphe (Fig 5 in FUXE 1965)	—	
3 The area just cranial to the nuc interpeduncularis mainly close to the ventral surface	scattered fine (NA)	3+ very fine
4 Nuc Darkschewitsch	1+ fine (NA)	—
5 Nuc interstitialis	1+ fine (NA)	—
Tectum		
1 Colliculus posterior	1+ very fine (NA)	scattered to 1+ very fine
2 Colliculus anterior	1+ to 3+ very fine (NA)	1+ to 2+ very fine
3 Pretectal region (see BUCHER and NAUTA 1954 and comments to Table 1)	1+ to 3+ very fine (NA)	1+ to 3+ very fine
Cerebellum (see comments to Table 1)	scattered to 1+ (NA)	—
Diencephalon		
I *Metathalamus*		
1 a) Nuc dorsalis corporis geniculati lateralis (See comments to Table 1)	3+ to 4+ very fine (NA)	1+ to 3+ very fine
b) Nuc ventralis corporis geniculati lateralis	1+ to 2+ very fine (NA)	2+ very fine

Table 1 (*cont*)

	Density of CA terminals	Density of 5 HT terminals
F Special somatic afferent nuclei		
1 Nuc cochlearis ventralis	2+ very fine (NA)	—
2 Nuc cochlearis dorsalis	scattered to 1+ very fine (NA)	— scattered fine
3 All vestibulary nuclei	—	
III *Other areas in the lower brain stem*		
A Medulla oblongata		
1 Substantia gelatinosa (Figs 64 65)	2+ fine (NA)	3+ very fine
2 Nuc gracilis	1+ very fine (NA)	1+ very fine
3 a) Nuc cuneatus medialis	1+ very fine (NA)	1+ very fine
b) Nuc cuneatus externus	scattered very fine (NA)	—
4 Inferior olivary complex		
a) Nuc olivaris inferior principalis (dorsal part) (Figs 2 20 in FUXE 1965)	5+ very fine (NA)	—
b) Nuc olivaris inferior principalis (ventral part) (Figs 2 20 in FUXE 1965)	2+ fine (NA)	scattered very fine
c) Nuc olivaris accessorius dorsalis (medial part) (Fig 2 in FUXE 1965)	1+ fine (NA)	scattered very fine
d) Nuc olivaris accessorius dorsalis (caudal lateral part)	scattered fine (NA)	dotted fluorescence of strong intensity
e) Nuc olivaris accessorius medialis (Figs 2 and 20 in FUXE 1965)	3+ to 4+ fairly thick (NA)	scattered very fine
B Other areas within the Pons		
1 Superior olivary complex	—	—
2 Nuc corporis trapezoidei	—	—
3 Nuc pontis (See comments to Table 1)	1+ to 3+ very fine (NA)	—
4 Substantia grisea periventricularis (see comments to Table 1 Fig 46)	1+ to 4+ fine (NA)	1+ to 4+ very fine
C Other areas within the mesencephalon Limbic midbrain area		
1 Nuc tegmenti dorsalis (mouse)	—	3+ very fine
2 Nuc tegmenti ventralis	3+ fine (NA)	—
3 The ventromedial part of the substantia grisea centralis (Fig 53)	3+ fine (NA)	—
4 The ventrolateral part of the substantia grisea centralis (see Fig 53)	5+ fine (mainly NA probably some DA)	—

Table 1 (*cont.*)

	Density of CA terminals	Density of 5-HT terminals
6 Nuc. periventricularis hypothalami (Fig. 41)	4+ to 5+ fine to fairly thick	scattered to 1+ very fine
7 The part just dorsal to the fornix	3+ to 4+ fine (NA)	scattered
8 The part ventral to the fornix between the nuc. ventromedialis and the nuc. lateralis hypothalami	5+ fine to fairly thick (mainly NA, probably some DA)	1+ very fine
9 Nuc. lateralis hypothalami	1+ to 3+ fine to thick (NA)	scattered to 1+ very fine
10 The retrochiasmatic area (see Figs. 38–50 and comments to Table 1)	4+ to 5+ fine to thick (mainly NA, probably some DA)	1+ to 2+ very fine
11 The external layer of the median eminence (see comments to Table 1 and FUXE, 1964)	dotted fluorescence of strong intensity (mainly DA, probably some NA)	—
12 Nuc. anterior hypothalami	1+ to 2+ fine (NA)	scattered to 2+ very fine
13 Nuc. paraventricularis	4+ to 5+ fine to fairly thick (mainly NA, probably some DA)	—
14 Nuc. supraopticus (Fig. 9 in FUXE, 1965)	4+ to 5+ fine mainly NA, probably some DA)	—
15 Nuc. suprachiasmaticus (see comments Table 1)	—	4+ very fine
16 Nuc. preopticus medialis (Fig. 34)	3+ to 4+ fine (NA)	scattered very fine
17 Nuc. preopticus lateralis (Fig. 34)	2+ to 4+ fine (NA)	—
18 Nuc. preopticus suprachiasmaticus (Fig. 34)	4+ fine (NA)	scattered very fine
19 Nuc. preopticus periventricularis (Fig. 34, Fig. 8 in FUXE, 1965)	4+ fine (NA)	—
20 Nuc. commissurae anterioris	3+ fine (NA)	—
Telencephalon		
I RHINENCEPHALON		
A Primary olfactory part	scattered very fine (NA)	—
B The limbic lobe		
a) Cortex		
1 Gyrus dentatus (See comments to Table 1, Figs. 47–48)	scattered to 4+ very fine (NA)	scattered very fine
2 Gyrus hippocampi (See comments to Table 1, Fig. 24 in FUXE, 1965)	few to 4+ very fine (NA)	scattered very fine

In the raphe region CA terminals make intimate contacts with a large number of the 5 HT nerve cells belonging to groups B1 B2 and B3 (see DAHLSTRÖM and FUXE 1964a) Many of the 5 HT cells of the nuc raphe dorsalis (group B7) are also in intimate contact with CA terminals The 5 HT cells in the nuc raphe pontis (group B5) and the nuc raphe medianus (group B8) on the other hand are only occasionally in contact with such terminals Together with the nuc linearis (pars caudalis intermedia and rostralis) the above mentioned raphe nuclei and those in the medulla oblongata form a well-defined anatomical complex i e *the raphe complex* (see TABER BRODAL and WALBERG 1960) In contrast to the other nuclei of the raphe complex no portion of the linear nucleus contains any 5 HT nerve cells In its caudal part there are found instead a large number of CA nerve cells (group A10)

CA terminals are present throughout the entire *reticular formation* making intimate contacts with non monoamine CA and 5 HT nerve cells alike In the ventrolateral part of the reticular formation in the medulla oblongata they make intimate contacts with the processes of nerve cells belonging to group A1 (Fig 1 in DAHLSTRÖM and FUXE 1964a) and in the ventromedial part of the reticular formation at the same level they make close contacts with some of the 5 HT nerve cells (Figs 30 31 in FUXE 1965) of group B1 B2 and B3 At this level the close contact with non fluorescent cells is particularly well demonstrated. These cells (Fig 21 in FUXE 1965) lie just dorsolateral to the nuc olivaris accessorius dorsalis

The CA terminals in the reticular formation range for the most part from scattered to medium density They are scattered for instance or very low in the paramedian part in the nuc reticularis pontis caudalis and oralis and in the parts of the mesencephalic reticular formation not mentioned below they are of low density in the nuc reticularis gigantocellularis the subnuc reticularis dorsalis medullae oblongatae and the nuc reticularis parvo cellularis and of medium density in the nuc reticularis lateralis and the subnuc reticularis ventralis medullae oblongatae They have a high to very high density however in the following two areas

1 The area surrounding the pedunculus cerebellaris superior (4+) especially in the dorsal part (5+) lateral to the nuc tr mesencephalici n trigemini (Figs 43 46)

2 The part of the mesencephalic reticular formation lying dorsal and dorsolateral to the lemniscus medialis at the level of the nuc interpeduncularis (this area largely corresponds to the cranial part of the cuneiform

area) Most of the CA nerve cells of group A8 are present in this area (Fig 6 in FUXE 1965) but only occasionally are the CA terminals seen to make intimate contacts with these cell bodies

5 HT terminals have so far been detected only in the reticular formation of the pons and the medulla oblongata Their density is usually very low and in some areas no 5 HT terminals at all are seen They are present mainly in the dorsomedial and ventromedial (Fig 57) part of the reticular formation in the medulla oblongata Intimate contacts made by 5 HT terminals with non fluorescent cell bodies are particularly well demonstrated in the area ventrolateral to the nuc hypoglossus (Fig 11 in FUXE 1965)

Both the *somatic* and the *visceral nuclei of the cranial nerves* contain monoamine terminals although the *visceral efferent* and *afferent* nuclei have a much higher density of both CA and 5 HT terminals In the *anterior horn* the *nuc hypoglossus nuc motorius n trigemini* and *nuc n facialis* some of the motor cells are observed to make intimate contacts with both types of terminals In the nuc motorius n trigemini a large number of the monoamine terminals make intimate contacts with the cell bodies (Figs 13 18 in FUXE 1965) while in the nuc tracti solitarii the majority of the terminals lie between the cell bodies without any close contact with them Electron microscopical studies on the nuc tracti solitarii (FUXE HOKFELT and NILSSON 1965) and the nuc motorius n trigemini (FUXE and HOKFELT unpublished data) also reveal that the vast majority of the synaptic contacts made in the former nucleus are axo dendritic while in the latter nucleus the motor cell bodies have abundant axo somatic junctions These findings strongly suggest that most of the monoamine terminals in the nuc tr solitarii form axo dendritic and axo somatic junctions respectively

Just dorsolateral to the *nuc ambiguus* there is a high accumulation of CA terminals (4+) In the mouse and hamster 5 HT terminals are present with a high density in the nuc ambiguus intimately surrounding its cell bodies

In the *substantia grisea periventricularis* of the fourth ventricle the 5 HT terminals are concentrated to the zone just under the ependyma mainly in the cranial midline part of the area The CA terminals are present mainly in the lateral part at the cranial end of the locus coeruleus medial to the nuc tr mesencephalici n trigemini

CA nerve cells of group A3 are found not only in the *nuc olivaris accessorius dorsalis* but also in the richly innervated *dorsal part* of the *nuc olivaris principalis* where very fine NA terminals form a very dense mesh work

In the *griseum pontis* a small region with a distinctly higher density of

NA terminals (3—) than the rest of this area is found in the nuc pontis medialis lying close to the midline in a dorsal position

There is little doubt that the unipolar cells of the *nuc tr mesencephalici n trigemini* some of which are in close contact with CA terminals are equivalents to peripheral ganglion cells which have developed inside the brain

Many of the 5 HT cells in the *limbic midbrain area* namely those present in the ventral part of the griseum centralis mesencephalici (these are mainly present in the nuc raphe dorsalis group B7) are in intimate contact with the CA terminals (see above) The 5 HT nerve cells in the nuc medianu raphe (group B8) however are in close contact with such terminal only occasionally and the same is true of the large number of CA nerve cells in the area ventralis tegmenti The nuclei tegmenti ventralis and dorsali which also belong to the *limbic midbrain area* contain no CA or 5 HT nerve cells but instead a fairly large number of NA and 5 HT terminal respectively

The area substantia nigra has a very low number of CA terminals mainly present in the zona compacta A mass of densely packed very fine 5 HT terminals seems to be present in the zona reticulata since a diffuse yellow fluorescence develops in this area after nialamide treatment The substantia nigra and especially the zona compacta of this area contain a large number of DA nerve cell (group A9) The nuc tractus opticus basalis however which is adjacent to the medial part of the zona compacta is completely devoid of both monoamine nerve cells and terminals

In the *tectum* the very fine NA and 5-HT terminals are found predominantly in the superficial strata The same is true of the distribution of CA terminals in the tectum optici in birds (FUXE and LJUNGGREN 1965) where they are much more frequent than in mammals

In the *pretectal region* the very fine 5 HT terminals are found predominantly in the area pretectalis medialis and the nuc pretectalis a very low density of NA terminals being also present The NA terminals are in a majority in the nuc tractus opticus pars lateralis and medialis

The number of very fine NA and 5-HT terminals present in the *nuc dorsalis corporis geniculati lateralis* in all mammals studied lie mainly between the cell bodies It seems probable that they are at least partly identical with the meshwork of fine terminal axon arborizations discovered by SZENTAGOTHAI (1963) in the lateral geniculate body of cat with the help of neurohistological methods Since these terminals were shown to make axo-dendritic contacts especially with the smaller dendrites the CA terminals in these regions probably form mainly axodendritic contacts Such plexa

of synaptic junctions are believed to have an inhibitory function in other parts of the central nervous system (SZENTAGOTHAI 1961) Since a considerable number of 5 HT terminals exist in the geniculate the fact that 5 HT in neurophysiological studies (BISHOP and DAVIS 1960 CURTIS and DAVIS 1962) has been shown to depress synaptic transmission in the geniculate is of great interest It may be that 5 HT functions as an inhibitory transmitter substance in the geniculate

The strong dotted green fluorescence observed in the *external layer of the median eminence* is due to the presence of very fine densely packed CA (mainly DA) terminals which converge to the primary capillary complex of the hypophyseal portal system (FUXE 1964) The CA rich zone in the external layer is best developed in the lateral part of the median eminence The internal layers have only a low to medium density Most of the CA terminals in these layers are fairly thick and a few are thicl

The retrochiasmatic area has a very high density of CA (mainly NA) terminals the majority of which have a horizontal direction and run more or less parallel to each other in the ventral supraoptic commissure of Gudden A large number of the CA terminals are fairly thick while others are thick They could in transverse sections 10 μ thick be traced up to 400 to 600 μ With the exception of the substantia gelatinosa in the spinal cord this is the only place where it has been possible to trace monoamine terminals for such a long distance The terminals make during their course contact with some nerve cell bodies but in view of the presence of a large number of horizontally oriented dendrites in this area (see SZENTAGOTHAI FLERKÓ MESS and HALÁSZ 1963) it seems very likely that the CA terminals to a large extent instead form parallel contacts with these dendrites It is possible that the terminals observed by the latter authors to make such contacts in Golgi preparations are at least partly identical with the CA terminals especially as they appear to have oval enlargements scattered along the fibre

Also in the ventral part of the dorsal supraoptic commissure (i e the commissure of Meynert) the CA terminals have essentially the same appearance The direction however is now observed in transverse section to be parallel to the inner contour of the optical tract The dendrites of the cells in this part also have this direction however and it thus seems very likely that the CA terminals also in this region form parallel axo dendritic junctions This picture is even more developed in the cat and dog

In the *nuc arcuatus* especially in its anterior portion and the ventral portion of the *nuc anterior periventricularis hypothalami* are found the small CA cells of group A12 Only occasionally however do the CA terminals in this region make intimate contacts with these cells

A small group of small closely packed weakly fluorescent CA nerve cells was found just dorsal to the nuc dorsomedialis hypothalami These have not been previously described and will therefore be referred to as group A13

The vast majority of the large cells in the *nuc supraopticus* and the *nuc paraventricularis* are in intimate contacts with CA terminals The terminals of the nuc supraopticus (see CARLSSON FALCK and HILLARP 1962) are most densely packed however in the ventral part and those of the nuc paraventricularis in the parvo cellular part In these parts most of the terminals seem to be in contact with processes not with cell bodies The part of the *nuc periventricularis hypothalami* just ventral to the nuc paraventricularis has a somewhat higher density of CA terminals than the rest of this nucleus

The ventromedial part of the *nuc preopticus medialis* has a high density of NA terminals the rest a medium density

The *nuc preopticus lateralis* has a low density of NA terminals except in the part situated somewhat ventrolaterally of the nuc interstitialis striae terminalis where the density is high

In the ventral part of the *nuc interstitialis striae terminalis* just ventral to the commissura anterior there is a very dense accumulation of numerous fine to fairly thick CA terminals which are observed to make intimate contacts with many cell bodies of this region A number of thick terminals are also observed to make such contacts

The *nuc suprachiasmaticus* is the only area of the hypothalamus in which a high density of 5 HT terminals has been found so far The terminals are concentrated in the ventral part of the nucleus Most of the remaining regions of the rat hypothalamus contain no or only scattered 5 HT terminals In the dog a low density of very fine 5 HT terminals is found in certain other parts of the hypothalamus such as the dorsal hypothalamic area and the nuc lateralis hypothalami and most regions of the dog hypothalamus have been observed to contain scattered 5 HT terminals

Just *medial to the dorsal part of the nuc accumbens* there is a small circumscribed area with a very strong dotted green fluorescence This area in all probability contains at least mainly DA Somewhat more ventromedially at the same level fairly close to the middle line and the ventral surface there are observed two other areas with a similar appearance lying like the first area at least partly within the nuc tractus diagonalis and also in all probability containing at least mainly DA

In the dorsal *hippocampus* there is a high density of CA terminals in the stratum radiatum of the CA 3 area (for terminology see GREEN 1964) while a very low to low density is observed in the stratum radiatum of the CA 1 and CA 2 areas The stratum lacunosum of these areas however contains a

medium density of CA terminals In the ventral hippocampus the stratum radiatum has a high density of NA terminals throughout The molecular layer of the hippocampus has a very low to low number of CA terminals They are present in very low numbers in the pyramidal layer of the hippocampus with the exception that such terminals are somewhat more frequent in the pyramidal layer of the CA 4 area

In the *gyrus dentatus* a high density of NA terminals is present in the zone just below the granular layer The granular and molecular layer on the other hand have only a very low to low density of NA terminals It seems probable that most of the NA terminals present in this zone and the stratum radiatum form axo dendritic junctions (*cf* BLACKSTAD 1963 see also FUXE 1965)

In the *cortex entorhinalis* and the *cortex piriformis* the very fine NA terminals are scattered or present in a very low to low density These seem mainly to make contacts with processes since most of them have no contacts with the cell bodies They are most frequent in the two outer layers In the *mesocortex* (*gyrus cinguli*) and the *neocortex* of *inter alia* the gyrus frontalis and parietale the terminals are found above all in the molecular layer In the deeper layers only few or no CA terminals have been observed Scattered 5 HT terminals are present in the superficial layers In the gyrus cinguli a medium density of NA terminals is present in the molecular layer No systematic study has been made on the neocortex but most gyri have been studied and so far only parts of the gyrus temporalis have been found to have another pattern of distribution of the NA terminals In these parts the pattern of distribution is the reverse with a medium density in the deeper layers and a very low density in the outer layers It may be that the primary auditory cortex also has this type of distribution pattern of the NA terminals The density of NA terminals in the neocortex is generally very low and in certain areas of practically all gyri they are almost completely lacking Especially the cortex terminals are observed much more distinctly if α methyl DOPA has been administered

No extensive studies have yet been made on the *cerebellar cortex* So far however only a very low density of CA terminals has been observed scattered in all layers In the *chicken cerebellum* which contains high concentrations of NA (PSCHEIDT and HIMWICH 1963) the density is medium to high A high density is seen in the entire granular layer while the other two layers show only a low to medium density The terminals seem mainly to make contact with processes but also with some cell bodies of *inter alia* the Purkinje cells

In the *reticular formation* there was observed besides terminals also a

tract of CA fibres with a weak to medium green fluorescence and mainly irregular medium fluorescent enlargements scattered at fairly regular intervals along the fibre. This tract can be traced from the medulla oblongata as far as the middle third of the mesencephalon. In the medulla oblongata it is seen to run just ventral to the nuc. tracti solitarii. Running upwards it passes between the pregenual fibres of the facial nerve and comes to lie in the ventral part of pedunculus cerebellaris superior. In the mesencephalon the fibres of the tract become scattered in the mesencephalic reticular formation. The tract consists at least partly of ascending fibres since a lesion in the cranial part of the mesencephalic reticular formation will cause an accumulation of catecholamines in these fibres as far down as the medulla oblongata (DAHLSTRÖM, FUXE, OLSON and UNGERSTEDT, unpublished data).

In the *subthalamus* there are present a large number of CA fibre bundles. Those in Forel's field H_2 are smooth and have a weak green fluorescence. At least most of these fibres pass into the medial forebrain bundle, giving rise to at least the majority of terminals in the hypothalamus, preoptic area and the other parts of the forebrain except for the DA terminals of the neostriatum (see below). The fibre bundles present in the ventromedial part of the crus cerebri at this level, which have the same appearance as those mentioned above, supply at least to a very large extent the neostriatum with DA terminals (ANDÉN, CARLSSON, DAHLSTRÖM, FUXE, HILLARP and LARSSON, 1964).

Within the *zona incerta* there are present a fairly large number of CA fibre bundles of the same appearance as those in the reticular formation. In the zona incerta the bundles are fairly scattered, but in the mesencephalon in the part just lateral to the nuc. n. oculomotorii they form a distinct tract. At the junction of the mesencephalon and diencephalon they abruptly pass ventrally to ascend—at least most of them—in the zona incerta. The direction of these fibres was determined by means of large lesions in the subthalamus (ANDÉN, DAHLSTRÖM, FUXE and LARSSON, unpublished data), which resulted in axotomy of the fibre bundles and a subsequent accumulation of CA in the proximal part of the fibres (see DAHLSTRÖM and FUXE, 1964c).

Among the cell bodies of the nuc. lateralis hypothalami is localized the *medial forebrain bundle*. A large number of 5-HT and CA fibres are present, the 5-HT fibre bundles being apparent only after nialamide treatment (50 to 500 mg/kg), when the smooth axons develop a fairly strong to strong yellow fluorescence (Fig. 65). Many bundles of 5-HT fibres lie medially of the CA fibres just lateral to the fornix. They can directly in the micro

scope be observed to arise from 5 HT cells of group B7 and B8 Most of the 5 HT fibres are scattered throughout the ventrolateral part of the medial forebrain bundle and a smaller tract of 5 HT fibres is present just ventro lateral to the retrolenticular portion of the capsula interna the fibres running more or less parallel to those in the medial forebrain bundle A large number of 5 HT fibres ascending mainly in the medial forebrain bundle turn medially and then run dorsally in the tractus diagonalis some of them bending caudally in the superficial part of the cingulum while others pass into the fimbriae hippocampi These two tracts probably supply the cingulate and superior cortex and the septum and the hippocampal formation with 5 HT terminals respectively

Summary of the distribution of the monoamine terminals in the central nervous system

CA terminals

The NA terminals have a wide spread distribution in the central nervous system from the cortex to the spinal cord The DA terminals on the other hand seem to be mainly concentrated to certain circumscribed areas in the forebrain

DA terminals

The DA terminals are accumulated mainly in the neostriatum and the tuberculum olfactorium They are in all probability accumulated also in the nuc accumbens and in small circumscribed areas medial to the nuc accumbens lying partly within the nuc tractus diagonalis Finally the external layer of the median eminence also contains a large accumulation of DA terminals In all these regions the DA terminals are very fine and so closely packed that the individual terminals are not observed the areas usually appearing in the micro scope with a diffuse or dotted green fluorescence The DA terminals probably answer for a major proportion of the CA terminals in the dorsolateral part of the nuc interstitialis striae terminalis and the nuc amygdaloideus centralis and a considerable part of the CA terminals in the nuc paraventricularis rotundo cellularis A number of DA terminals are probably present also in certain nuclei of the lower brain stem and in the hypothalamus See Table I

NA terminals

Spinal cord (see Dahlström and Fuxe 1965)

Lower brain stem The NA terminals are found in high to very high densities in many of the visceral afferent and efferent nuclei e g the nuc tracti solitarii the nuc motorius dorsalis n vagi the nuc motorius n trigemini in the nuc raphe pallidus the nuc raphe obscurus in the dorsal limb of the nuc olivaris principalis in certain parts of the reticular formation (see comments to Table I) and in the ventrolateral part of the griseum centralis mesencephalici which belongs to the limbic midbrain area The majority of the regions in the lower brain stem however have a very low or low density of NA terminals e g all the somatic afferent and efferent nuclei except the nuc hypoglossus

large parts of the reticular formation the raphe complex in the pons and mesencephalon except the nuc raphe dorsalis and the sensory nuclei of the second and third order (the nuc gracilis nuc cuneatus and the colliculus anterior and posterior) In some areas practically no CA terminals at all are observed e g in the vestibulary nuclei the superior olivary complex the nuc corporis trapezoidei the nuc ruber and large parts of the substantia nigra area the two latter areas belonging to the extrapyramidal motor system

Diencephalon A high to very high density of NA terminals is observed in many hypothalamic nuclei e g nuc dorsomedialis hypothalami, nuc paraventricularis nuc supraopticus nuc periventricularis hypothalami and nuc lateralis hypothalami and many hypothalamic areas e g the retrochiasmatic area and the preoptic area (above all the nuc preopticus suprachiasmaticus and the nuc preopticus periventricularis) The majority of nuclei in the mammillary body and the thalamus on the other hand have a very low to low density of NA terminals In the vegetative hypothalamus the nuc ventromedialis hypothalami and nuc hypothalami anterior are the only nuclei to have a very low to low density of NA terminals and the nuc suprachiasmaticus has none at all The nuc anterior ventralis thalami and nuc paraventricularis rotundocellularis have a very high density of CA terminals

Telencephalon The NA terminals are to a very large extent distributed among various regions of the limbic lobe Considerable numbers of NA terminals are found e g in the hippocampal formation the amygdaloid cortex the gyrus cinguli the nuc septalis lateralis and in the ventral part of the nuc interstitialis striae terminalis In other areas of the limbic lobe there are large numbers of DA terminals (see above) The neocortex and the primary olfactory part of the rhinencephalon on the other hand contain only a relatively small number of CA terminals

5 HT terminals

Fairly large numbers of 5-HT terminals have been found in the lower brain stem and especially in the spinal cord while in the diencephalon and the telencephalon only some nuclei have been found to contain considerable numbers of 5-HT terminals e g the nuc suprachiasmaticus the superficial part of the nuc amygdaloideus medialis and the pretectal region This in spite of treatment with a large dose of nialamide in normal or reserpinized animals Since fairly high concentrations of 5 HT have been found in e g the hypothalamus and the neostriatum (0 7 μg/g 0 5 μg/g ANDÉN unpublished data) this is in all probability due to the fact that the 5 HT terminals are very fine many of them probably sub lightmicroscopical Furthermore their fluorescence rapidly decreases in intensity on UV irradiation which makes them still more difficult to discover If the very fine 5 HT terminals however are densely packed as in e g the nuc suprachiasmaticus then they are fairly easily visualized especially if no CA terminals are present

In the lower brain stem a medium high or very high density is found *inter alia* in many of the visceral efferent and afferent nuclei (e g nuc motorius n trigemini nuc motorius n facialis nuc motorius dorsalis n vagi nuc salivatorius superior and nuc intercalatus) in the cranial part of the substantia grisea periventricularis just under the ependyma in an area just dorsal to the caudal

half of the nuc interpeduncularis and ventral to the nuc medianus raphe in the substantia gelatinosa in the area just under the cells of the subcommissural organ and in the ventrolateral part of the nuc olivaris accessorius dorsalis

In the spinal cord a very high and a medium density are found respectively in the dorsolateral part of the sympathetic lateral column and in the sacral para sympathetic nucleus The 5 HT terminals are of medium to high density in the ventral horn In the lumbosacral part the density is mostly very high

Essentially the same type of distribution of the monoamine terminals has been found also in the other mammals studied

Schematic illustrations on the distribution of monoamine nerve terminals in the brain

In Textfigures 1–13 the CA terminals are indicated to the left by dots and the 5 HT terminals are indicated to the right by crosses in Textfigures 1–7 Their topography and relative density are shown The Textfigures are schematic drawings made on the basis of the photographs and drawings in the books of Zeman and Innes 1963 (Textfigs 1–4) and of König and Klippel 1963 (Textfigs 5–13)

Abbreviations used in textfigures

A

A nuc accumbens
ABL nuc amygdaloideus basolateralis
AC aqueductus cerebri
ACE nuc amygdaloideus centralis
ACO nuc amygdaloideus corticalis
ALP nuc amygdaloideus posterolateralis
AM nuc amygdaloideus medialis
AMB nuc ambiguus
AP area postrema
ARC nuc arcuatus

B

BCI brachium colliculi inferioris

C

C nuc commissuralis
CA commissura anterior
CAA commissura anterior pars anterior
CAI capsula interna
CAIR capsula interna pars lenticularis
CAP commissura anterior pars posterior
CC crus cerebri
CCGM nuc centralis corporis geniculati medialis
CF columna fornici
CFV commissura fornicis ventralis
CG cingulum
CI colliculus inferior
CL claustrum
CO chiasma opticum
CP commissura posterior
CPF cortex piriformis
CPU nuc caudatus putamen
CSDV commissura supraoptica dorsalis pars ventralis
CT corpus trapezoideum

D

D nuc Darkschewitsch
DCGL nu dorsalis corporis geniculati lateralis

E

EM eminentia mediana
EWC nuc Edinger Westphal pars caudalis

F

F fornix
FH fimbria hippocampi
FL fasciculus longitudinalis
FLM fasciculus longitudinalis medialis
FMP fasciculus medialis prosencephali
FMT fasciculus mammillo thalamicus
FMTG fasciculus mammillo tegmentalis
FOP fasciculus opticus
FOR formatio reticularis
FR fasciculus retroflexus
FS fasciculus solitarius

G

G nuc gelatinosus
GC griseum centralis
GCC genu corpora callosi
GCI gyrus cinguli
GD gyrus dentatus
GNF genu n facialis
GP griseum pontis
GPA globus pallidus

H

H_1 Forel field H_1
H_2 Forels field H_2
HA nuc anterior hypothalami
HD nuc dorsomedialis hypothalami
HI gyrus hippocampi
HL nuc lateralis hypothalami
HP *nuc hypothalamicus posterior*
HPV nuc periventricularis hypothalami
HVM nuc ventromedialis hypothalami

I

I nuc interstitialis
IC insulae Calleja
ICM insula Calleja magna
IP nuc interpeduncularis

L

LC nuc linearis pars caudalis
LH nuc lateralis habenulae
LM lemniscus medialis

M

MCGM nuc marginalis corporis geniculati medialis
MH nuc medialis habenulae
MP nuc mammillaris posterior
MPL nuc mammillaris prelateralis
MR nuc medianus raphe
MML nuc mammillaris medialis pars lateralis
MMM nuc mammillaris medialis pars medialis

N

NIII nuc n oculomotorii
NXII nuc hypoglossus
NC nuc cuneatus
NCD nuc cochlearis dorsalis
NCT nuc corporis trapezoidei
NCV nuc cochlearis ventralis
NDM nuc motorius dorsalis n vagi
NG nuc gracilis
NIC *nuc intercalatus*
NMT nuc motorius n trigemini
NNF nuc n facialis
NOAD nuc olivaris accessorius dorsalis
NOAM nuc olivaris accessorius medialis
NOPD nuc olivaris principalis pars dorsalis
NOPV nuc olivaris principalis pars ventralis
NPRT nuc principalis n trigemini
NR nuc reticularis gigantocellularis
NRC nuc reticularis pontis caudalis
NRL nuc reticularis lateralis
NRP nuc reticularis paramedialis
NRPC nuc reticularis parvocellularis
NRT nuc reticularis thalami
NRV subnuc reticularis ventralis medullae oblongatae
NTP nuc tegmenti pontis
NTR nuc tractus spinalis n trigemini

O

OS nuc olivaris superior

P

P nuc pretectalis
PCI pedunculus cerebellaris inferior
PCM pedunculus cerebellaris medius
PCS pedunculus cerebellaris superius
PF nuc parafascicularis
POL nuc preopticus lateralis

POM nuc preopticus medialis
POP nuc preopticus periventriculari
POSC nuc preopticus suprachia mati cus
PP nuc pretectalis profundus
PT nuc parataenialis
PV nuc premammillaris ventralis
PVM nuc paraventricularis pars mag nocellularis
PVP nuc paraventricularis parvocellu lari
PVR nuc paraventricularis rotundo cellularis
PVS nuc paraventricularis stellato cellularis

R

R nuc ruber
PCC radiatio corporis callosi
RC retrochiasmatic area
RD nuc raphe dorsali
PE nuc reuniens
RH nuc rhomboideus
RM nuc raphe magnu
RO nuc rahpe obscuru
RP nuc raphe pallidu
RPI recessus p nealis
RTRS radix tractus spinalis n trigemini

S

S nuc suprageniculatu
SC nuc suprachiasmat cu
SF nuc septalis fimbriali
SG substantia gelatinosa
SGP substantia grisea periventricularis
SGS stratum gri eum superfi ial coll culi superioris
SL nuc septalis laterali
SM striae medullares
SME nuc septi mediali
SNC substantia nigra zona compacta
SNR substantia n gra zona reticulata
SO nuc supraopticu
SOL nuc tracti solitariu
SR sulcu rhinali
ST nuc interstitialis striae terminalis
STR nuc triangularis septi
SUM area supramammillaris
SUT nuc subthalamicus

T

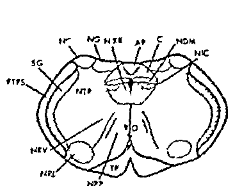
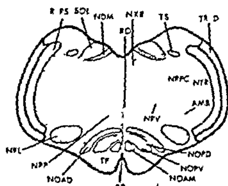
TAD nuc anterior dorsalis thalami
TAM nuc anterior medialis thalami
TAV nuc anterior ventralis thalami
TCC truncus corporis callosi
TD nuc tractus diagonalis
TL nuc lateralis thalami
TLP nuc lateralis thalami pars poste rior
TM nuc medialis thalami
TO tractus opticus
TOL tractus olfactorius lateralis
TOP nuc tractus optici pars lateral
TOM nuc tractus optici pars mediali
TP tractus pyramidalis
TPO nuc posterior thalami
TPT tractus pedunculo tegmentalis
TRD tractus diagonalis
TRS tractus rubro-spinalis
TRSD tractus spino-cerebellaris dor salis
TS tractus solitarius
TUB tuberculum olfactorium
TV nuc ventralis thalami
TVD nuc ventralis thalami pars dorsa lis
TVM nuc ventral s medialis thalami pars magnocellularis

V

VIII ventriculus tertii
VC vestibulary complex
VCGL, nuc ventralis corporis geniculati lateralis
VCLL, nuc ventralis caud ili l mni i laterali
VL ventriculus laterali

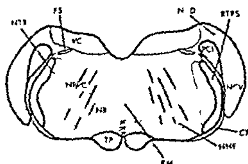
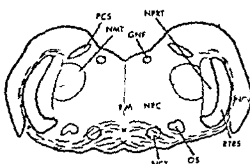
Z

ZI zona incerta

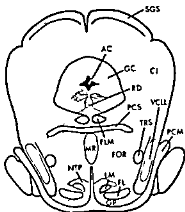
TEXT FIG 1 Transverse section of the brain stem at the level of the inferior third of the inferior olive

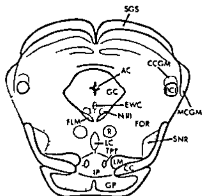
TEXT FIG 2 Transverse section of the brain stem at the rostral third of the olivary complex

TEXT FIG 3 Transverse section of the brain stem at the level of the maximal development of the nuc n facialis

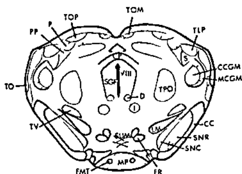
TEXT FIG 4 Transverse section of the brain stem at the level of the nuc motorius n trigemini

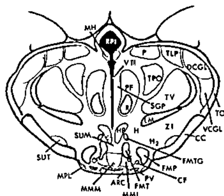




TEXT FIG 5 Transverse section of the brain stem at the level of the inferior colliclе

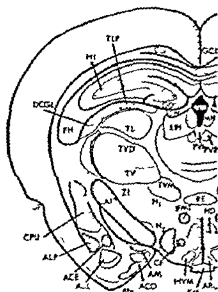
TEXT FIG 6 Transverse section of th st m at the level of the nuc interpedun cularis

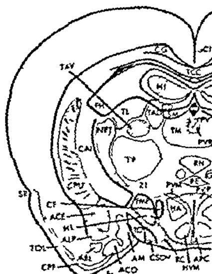




TEXT FIG 7 Transverse s ct on of the brain stem at the level of the commissura posterior

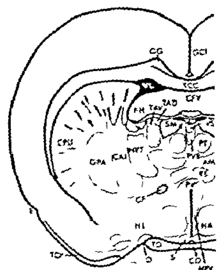
TEXT FIG 8 Transverse section of th brain stem at the level of the corpus mammil lare

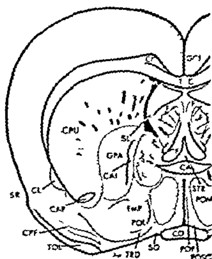




TEXT FIG 9 Transverse section of the brain stem at the level of the posterior part of the median eminence

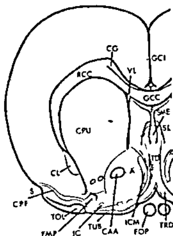
TEXT FIG 10 Transverse section of the brain stem at the level of the nuc para ventricularis hypothalami





TEXT FIG 11 Transverse section of the brain stem at the level of the posterior part of the chiasma opticum

TEXT FIG 12 Transverse section of the brain stem at the level of the preoptic area



TEXT FIG 13 Tran verse section of the telencephalon at the level of the rostral part of the septum

II Effects of drugs interfering with monoamine metabolism

The results from these experiments involving *inter alia* the administration of reserpine MAO inhibitors and DOPA analogues have been partly reported in an earlier paper (FUXE 1965) The following account of the reaction of the monoamine terminals to these drugs will be more comprehensive and is especially concerned with the differences between the various terminal systems

Reserpine

The time course and do e dependence of the various terminal systems of the brain were studied A supramaximal dose (10 mg/kg) was used in the time course experiment In the dose response experiment two fixed time intervals (4 and 24 hours) were used

Certain data on the time-course and dose dependence have already been given (FUXE 1965) particularly as regards the hypothalamic NA terminals (see CARLSSON FALCK and HILLARP 1962) No other changes were found than a successive disappearance—at varying speeds—of the specific fluorescence followed by a gradual recovery to normal levels For doses times and number of animals see Table II

A *Time course of depletion and recovery* (10 mg/kg)

After 30 minutes only the terminals of the median eminence showed any noticeable reduction in intensity The terminals in all other regions seemed to be un affected

After 1 hour a very strong decrease was observed in the fluorescence intensity of the CA terminals present in the median eminence and a strong decrease in

TABLE II *Time before killing after the first injection*

Number of animals within parentheses

Drug	Dose mg/kg body wt	Hours
Reserpine i.p.	10	½(6) 1(7) 2(7) 3(4) 4(8) 6(6) 8(8) 12(6) 24(10) 48(6) 72(6) 96(7) 9[illegible]
	5	24(13)
	1	4(6) 24(6)
	0.5	4(5) 24(5)
	0.1	4(6) 24(6)
	0.05	4(6)
Nialamide i.p.	500	1(4) 2(5) 4(6) 5–6(50) 8(4) 16(3) 24(5) 48(4)
	250	5–6(4)
	100	4(4)
	50	5–6(4)
Reserpine i.p. + Nialamide i.p.	10 500	24 5–6(25)
MO 911 i.p.	300	1(5) 2(5) 3(5) 4–5(8)
	400	3–5(8)
Iproniazide i.v.	200	4(3) 12(3) 17(8)
Nialamide i.p. + L DOPA s.c.	500 100	5–6 ½(6) 1(10)
Reserpine i.p. + Nialamide i.p. + L DOPA s.c.	10 100 50	6 3 ½(12)
Nialamide i.p. + DL 5 HTP s.c.	500 75	5–6 1(6)
Nialamide i.p. + DL 5 HTP s.c.	50 100	3½ ½(5)
m-Tyrosine i.p. 3	400	6(12) 8(4) 10(4) 12(4) (3 doses with 2 h intervals)
α-Methyl-*m*-tyrosine i.p.	400	24(12) 48(4) 96(5)
2	400	9(6) 12(6) 15(6) 18(6) 24(10) (2 doses with a 2 h interval)
α-Methyl-DOPA i.p.	400	24(6)

intensity had occurred in the fluorescence of the neostriatum, the nuc. accumbens and above all the tuberculum olfactorium. A strong to very strong reduction in both intensity and number was found in the areas containing mainly or exclusively very fine terminals (e.g. the gyrus cinguli, neocortex, nuc. reticularis thalami, tectum, nuc. suprachiasmaticus, nuc. pontis, nuc. cochlearis ventralis, the hippocampal formation, parts of the reticular formation, nuc. anterior ventralis thalami and the dorsal limb of the nuc. principalis olivaris inferior). Many richly innervated nuclei and areas, especially in the medulla oblongata and

pons contain for the most part fine terminals e g the ventral and dorsal horn nuc motorius n trigemini nuc n facialis nuc hypoglossus nuc of Edinger Westphal nuc salivatorius superior and nuc supraopticus The terminals in these areas showed a strong decrease in number and intensity after 1 hour Many nuclei and areas especially in the hypothalamus showed only a small decrease in number and intensity e g the ventro lateral part of the griseum centralis the nuc paraventricularis the ventral part of the nuc interstitialis striae terminalis the retrochiasmatic area the lower raphe nuclei and the nuc periventricularis hypothalami A large proportion of the monoamine terminals in these nuclei are fairly thick The nuc motorius dorsalis n vagi nuc tr solitarii and sympathetic lateral column showed a medium decrease in number and intensity

After 2 hours the median eminence was practically empty of fluorescence The neostriatum nuc accumbens and tuberculum olfactorium showed a very strong reduction in fluorescence intensity the neostriatum and above all the tuberculum olfactorium being most affected In the very strong reduction category were also the nuclei and the regions with mainly very fine terminals Somewhat less marked changes were found in the areas with a dotted type of fluorescence (except for the median eminence see above) e g the nuc amygdaloideus centralis and the dorso lateral part of the nuc interstitialis striae terminalis and the areas lying partly within the nuc tr diagonalis the latter being somewhat less affected than the former two A large to very large reduction in both number and intensity was obtained in the areas containing mainly fine terminals In many areas in which the majority of the terminals were fine to fairly thick the CA terminals showed a medium decrease in number and in intensity A strong to very strong decrease in these respects was obtained however in the nuc motorius dorsalis n vagi the nuc tractus solitarii and the sympathetic lateral column

After 4 hours practically no terminals of the very fine type were observed and only a very weak fluorescence intensity remained in the areas with diffuse fluorescence A very large decrease in fluorescence intensity had occurred in the *areas with a dotted fluorescence In many areas or nuclei containing mainly* fine terminals there remained only a small number of weakly fluorescent terminals The same was true in the main also of the fine terminals in nuclei where a considerable proportion of the monoamine terminals are fairly thick In many areas and nuclei (e g in the nuc paraventricularis especially in the parvocellular part in the ventral part of the nuc interstitialis striae terminalis in the caudal raphe nuclei and in the retrochiasmatic area) most of the fairly thick terminals were still fluorescent although usually with only a weak to medium intensity In the nuc motorius n vagi nuc tr solitarii and sympathetic lateral column however only a small proportion of terminals still exhibited any fluorescence The vast majority of thick terminals however which are present in the nuc preopticus lateralis especially the dorsal part the ventral part of the nuc interstitialis striae terminalis the nuc lateralis hypothalami the retrochiasmatic area the subthalamus the amygdaloid cortex and the reticular formation of the mesencephalon showed a small reduction in fluorescence intensity

After 6 hours only a small number of the fairly thick CA terminals were observed with a weak to medium fluorescence e g in the retrochiasmatic area and in the ventral part of the nuc interstitiales striae terminalis The number

and intensity of the thick terminals were strongly decreased After 8 to 24 hours most of the thick terminals had disappeared but a very small number remained with a weak to medium fluorescence intensity No fairly thick terminals were observed

Regeneration started after 3 to 4 days with the appearance of fine weakly fluorescent varicosities especially in the areas rich in monoamine terminals and was most rapid in the median eminence and the areas with a diffuse green fluorescence which are in all probability rich in DA terminals Even 10 days after the reserpine injection however recovery was still far from complete in many areas The thick terminals all showed a normal fluorescence intensity while the rest of the terminals were still considerably reduced in number and intensity in many regions of the brain

The time course of depletion and recovery of the 5 HT and CA terminals was essentially the same It must be pointed out however that since practically all 5 HT terminals are very fine to fine the terminals remaining after 8 hours were practically all CA Furthermore the 5 HT terminals in the nuc suprachiasmaticus had a very slow recovery and were barely visible 10 days after the injection of reserpine (FUXE and MEYERSON to be published)

B *Dose response experiment* (Time 24 h)

With certain samples of Serpasil the dose dependence was found to be somewhat different from that described below After doses of 0 05 mg/kg and 0 1 mg/kg the number and fluorescence intensity of the terminals seemed to be fairly unchanged The only effect observed was a weak decrease in the fluorescence intensity of the median eminence after a dose of 0 1 mg/kg

After a dose of 0 5 mg/kg a strong decrease in fluorescence intensity was obtained in the median eminence The areas with a diffuse fluorescence above all the tuberculum olfactorium showed a medium decrease in intensity as did those containing mainly or exclusively very fine terminals In the latter areas the number was markedly reduced In the areas with a dotted fluorescence a weak decrease in intensity was observed Areas in which the fine terminals are in a majority showed a weak decrease in both number and intensity while areas where the fairly thick terminals form a considerable proportion of the terminals showed no obvious change

After a dose of 1 mg/kg the external layer of the median eminence was completely depleted of amines The areas with a diffuse fluorescence showed a large to very large reduction in intensity and the areas with a dotted fluorescence (other than the median eminence) showed a medium to strong reduction In the areas containing mainly or exclusively very fine terminals there remained only a few scattered terminals with a weak green fluorescence and in areas where the fine terminals are in a majority only a small to medium number of terminals with a weak to strong fluorescence Areas with a considerable proportion of fairly thick terminals showed a considerable decrease in the number and intensity of terminals many terminals of a normal fluorescence intensity however were still observed The terminals in the nuc motorius dorsalis n vagi and nuc tractus solitarii were much more affected than those in e g the ventral part of the nuc interstitialis striae terminalis the ventrolateral part of the griseum centralis and the nuc paraventricularis This in spite

of being of the same size range as the terminals in the latter regions The terminals in the ventral part of the nuc interstitialis striae terminalis were found in contrast to terminals with the same size range in other areas and nuclei to be more or less unaffected by a dose of 1 mg/kg

After doses of 5 and 10 mg/kg the only terminals to be seen were a very small number of thick terminals of a weak to medium green fluorescence

(Time 4 h)

The effects of varying doses were studied 4 hours after the administration of reserpine Results were essentially the same as those obtained in the 24 hours experiment described above although the intensity and number of terminals after each dose was somewhat less decreased as compared with the results from corresponding doses in the 24 hours experiment

Fairly large differences thus exist between the various monoamine terminal systems of the central nervous system in rates of depletion after *reserpine and in the degree of sensitivity to reserpine These differences* are of importance in biochemical determinations of the amine contents in the brain of the reserpine treated animal At a certain time after reserpine administration the biochemical value will reflect a mean value of the amine contents present in the various monoamine terminal systems some of which will be markedly depleted while others have a fairly normal amine content

It must be pointed out however that the large differences observed between various CA terminal systems in respect of depletion and recovery rates or sensitivity to reserpine are partly correlated to different thickness of the varicosities of the terminals in the respective systems This might be partly due to the fact that the decrease in the amine contents of the very fine to fine terminals is more readily visualized than in the fairly thick to thick terminals It may well be however that the finer terminals which show a rapid depletion have a higher turnover than the thicker terminal since the same time course of depletion has been observed between the various systems of CA terminals after administration of a very potent inhibitor of the CA synthesis (H 44/68 generously supplied by Dr H Corrodi) The very fine DA terminals would thus seem to have a high metabolic turnover as appears to be the case also with the 5 HT terminals which are likewise mainly very fine

The fact that the terminals in one and the same nucleus *e g* the nuc supraopticus and the nuc motorius n trigemini showed large differences in depletion recovery and sensitivity to reserpine in spite of having the same general thickness clearly shows that other factors must be involved Similar findings have been obtained also after the administration of

H 44/68 (unpublished data) It may well be that the terminals within an apparently homogeneous population have markedly different rates of amine turnover this could be due to differences in impulse flow (SEDVALL 1964) although other factors are not excluded (cf NILSSON 1964)

MAO inhibitors

Various MAO inhibitors have been shown to increase markedly to very markedly the amine levels of the 5 HT neurons while the CA neurons are much less affected (DAHLSTRÖM and FUXE 1964*a* BAPTONÍČEK DAHLSTRÖM and FUXE 1964) In the present paper the various 5 HT terminal systems have been studied at varying times after the administration of different doses of nialamide and MO 911 (isonicotinyl benzylcarbamyl ethylhydrazine and N benzyl N methyl propanylamine)

With a dose of 500 mg/kg nialamide the increase in the fluorescence intensity of the 5 HT terminals could be observed after about 2 hours, was fully developed after 4 to 6 hours and persisted fairly unchanged during the following 24 hours The fluorescence intensity then showed a slow return to normal levels No obvious differences were observed between the different systems of 5 HT terminals in the brain or between the 5 HT terminals in one and the same system With a dose of even 50 mg/kg of nialamide the terminal systems showed a marked increase in intensity (5 to 6 hours after an i p injection) All doses used and especially the higher doses gave a clear increase also in the number of visible 5 HT terminals in all parts of the brain 4 to 6 hours after injection MO 911 gave essentially the same results as nialamide Iproniazide (N isopropyl N isonicotinylhydrazine) gave a much less marked increase in the amine contents of the 5 HT neurons (see also DAHLSTRÖM and FUXE 1964*a*) The highest accumulation of 5 HT following the administration of this drug was observed after 12 hours at which time a medium increase in intensity and a clear increase in the number of the 5 HT terminals was observed For doses and times see Table II

With the highest doses of MAO inhibitors—especially if the animals were kept at a temperature of +29 C–+35 C—there were seen fluorescence microscopical signs of amine release (or leakage) from both the 5 HT and CA terminals This will be discussed in another paper (CARLSSON DAHLSTRÖM FUXE and LINDQVIST 1965)

Reserpine Nialamide

As previously reported nialamide causes in reserpine treated animals a rapid accumulation of amines in the 5 HT neurons but not in the CA neurons (DAHLSTRÖM and FUXE 1964*a* FUXE 1965) which is in good agreement with the biochemical findings (CARLSSON LINDQVIST and MAGNUSSON 1960) This procedure is one of the best ways of studying the 5 HT terminals since no CA terminals are left to disturb the fluorescence microscopical picture About the

same marked increase was obtained in the various systems of 5 HT terminals The terminals showed about the same appearance as in normal animals —The same findings were made in experiments with mice

Reaccumulation of 5 HT occurred even with a small dose of nialamide (50 mg/kg) given to animals pretreated (2 hours) with a large dose of reserpine (5 mg/kg) (DAHLSTROM FUXE and HILLARP 1965)

Nialamide DOPA

The increase obtained in the CA levels of the brain by the administration of L DOPA after MAO inhibition (CARLSSON LINDQVIST MAGNUSSON and WALDECK 1958 BERTLER and ROSENGREN 1959 CARLSSON 1959 CARLSSON and HILLARP 1962) seems to be due at least partly to increased amine levels in the cell bodies axons and terminals of the CA neurons (see FUXE 1965) In reserpinized animals distinctly visible CA terminals reappeared after this treatment Both in normal and reserpinized animals markedly increased fluorescence intensities were observed in the neostriatum and in the other DA containing areas with a diffuse fluorescence

Neither the terminals nor the cell bodies of the 5 HT neurons showed any clear accumulation of CA (or DOPA) after this treatment in normal or reserpinized rats except in some cases in which the 5 HT cells of group B 1 had developed a very weak green fluorescence

Nialamide 5 HTP

After MAO inhibition the administration of 5 HTP results in very strongly increased 5 HT levels in the brain (UDENFRIEND WEISSBACH and BOGDANSKI 1957) In a previous paper (DAHLSTROM and FUXE 1964*a*) no certain changes in the 5 HT neurons were observed after the administration of 5 HTP in nialamide treated animals This however was in all probability due to the use of an excessive dose of nialamide which in itself produces a very marked increase in the amine levels If 5 HTP was administered 3 h after a small dose of nialamide (50 mg/kg) a clear increase was observed in the fluorescence of the cell bodies non terminal axons and above all the terminals of the 5 HT neurons The increase in 5 HT levels in the brain is thus at least partly ascribable to increased amine contents in these neurons

Neither the terminals nor the cell bodies of the CA neurons showed any clear accumulation of 5 HT (or 5 HTP) after this treatment in normal or reserpinized animals

Since there is good evidence that most of the DOPA/5 HTP decarboxylase is localized to and has a high activity in the monoamine neurons (ANDEN 1965 and unpublished data ANDEN MAGNUSSON and ROSENGREN 1965) the increased fluorescence intensity observed in these neurons after

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were for the most part still clearly reduced in intensity The thick terminals however now had a normal fluorescence intensity as had many of the terminals in the areas which 2 hours after the last dose contained terminals of a weak green fluorescence After 6 to 8 hours a full recovery in respect of both intensity and number of the CA terminals had occurred in all regions The CA cell bodies of the CA neurons showed about the same time course of depletion and recovery (see DAHLSTRÖM FUXE and HILLARP 1965)

α methyl m tyrosine

In a previous paper (FUXE 1965) strong support was obtained for the view that the CA terminals showing a rapid recovery after α MMT administration are DA terminals while those with a slow recovery are NA terminals (see also CARLSSON 1964) In the present investigation the NA terminals showed a gradual recovery to reach normal levels only after several days After an administration of one dose of α MMT these levels are reached in the brain and the spinal cord after about 4 days This is in complete agreement with the biochemical data also obtained in rats (ANDEN 1964) After two days recovery in the NA terminals had started in most parts of the brain and in the spinal cord No marked differences between the various systems of terminals were observed

Tetrabenazine

Tetrabenazine which is chemically quite different from reserpine gives a rapid but short lasting depletion of the brain monoamine stores (see PLETSCHER BROSSIE and GEY 1962 CARLSSON 1965) This drug has in a previous paper (BAPTONIČEK DAHLSTRÖM and FUXE 1964) been reported completely to abolish the fluorescence of CA and 5 HT terminals in 3 hours followed by complete recovery within 20 hours Further studies have revealed that both the terminals and cell bodies of the CA neurons show a distinct recovery as early as after 8 to 12 hours (DAHLSTRÖM FUXE and HILLARP 1965) No obvious differences in recovery rates have been observed between the various CA and 5 HT terminal systems

GENERAL DISCUSSION

There is very strong evidence that the monoamine terminals in the central nervous system are true synaptic terminals and that the varicosities which have extremely high amine concentrations, represent the presynaptic structures specialized for the synthesis storage and release of the amines The evidence for the concept that the monoamines are neurohumoral transmitters has been obtained both from physiological (ANDÉN CARLSSON HILLARP and MAGNUSSON 1964 1965) neurophysiological (ANDÉN LUNDBERG ROSENGREN and VYCLICKY 1963, ANDÉN JUKES and LUNDBERG 1964 ANDÉN JUKES LUNDBERG and VYCLICKY 1964) and histochemical studies (DAHLSTRÖM and FUXE 1964a FUXE and GUNNE 1964 DAHLSTRÖM FUXE KERNELL and SEDVALL 1965 FUXE 1965)

The exact origins of the different DA NA and 5 HT terminal systems in the central nervous system are still to a large extent unknown even if those of several large systems have been established by means of the histochemical fluorescence method Strong evidence has been given for the view that the NA and 5 HT terminals in the various parts of the spinal cord originate from descending fibres which belong at least mainly to CA and 5 HT cell groups in the medulla oblongata i e groups A1–A2 and groups B1–B3 respectively (DAHLSTRÖM and FUXE 1964a 1965) Further evidence for this view is given by the fact that very large unilateral lesions in the mesencephalon which destroy the large CA groups A8 A9 and A10 on one side do not affect the appearance of the NA terminals in the spinal cord (DAHLSTRÖM FUXE OLSON and UNGERSTEDT 1964)

The origins of the vast number of DA terminals in the neostriatum have recently been demonstrated (ANDÉN CARLSSON DAHLSTRÖM FUXE HILLARP and LARSSON 1964 ANDÉN DAHLSTRÖM FUXE and LARSSON 1965a 1965b) They were found to belong to a large nigroneostriatal DA neuron system

There exist also other large ascending CA neuron systems from the lower brain stem to different parts of the diencephalon and telencephalon This has been shown by unilateral lesions in the cranial ventral part of the mesencephalon which destroy for the most part the large CA cell groups A8 and A10 (DAHLSTRÖM FUXE OLSON and UNGERSTEDT 1964) and by

unilateral lesions of the medial forebrain bundle with or without involvement of the crus cerebri (ANDEN DAHLSTROM FUXE and LARSSON 1965b) 10 to 21 days after large unilateral lesions involving mainly the medial forebrain bundle at the mammillary level the DA terminals in the tuberculum olfactorium and the nuc accumbens had completely disappeared Furthermore the CA terminals in the so called limbic forebrain structures (amygdaloid cortex hippocampal formation septal area) in the gyrus cinguli in the neocortex in the preoptic area in the nucleus interstitialis striae terminalis and in most parts of the hypothalamus showed a marked to very marked decrease in numbers These results give clearcut evidence for the view that the vast majority of the CA terminals outside the neostriatum are derived from predominantly ipsilateral systems of ascending CA fibres at least mainly present in the medial forebrain bundle Since first increased and then decreased amine levels together with degenerative changes have been observed in the very large CA group A10 after lesion of this bundle and while group A10 is known to send fibres to the medial forebrain bundle (DAHLSTROM and FUXE 1964a) there is almost conclusive evidence for the existence of large ascending CA—mainly NA—neuron systems *e g* mesencephalo hypothalamic mesencephalo limbic and mesencephalo cortical systems Some crossing probably takes place caudal to the level of the mammillary complex since somewhat less marked changes occur in the terminals on the ipsilateral side after destruction of groups A8 to A10 than after large unilateral lesions of the medial forebrain bundle at the levels mentioned above

After leaving the medial forebrain bundle the CA fibres to the hippocampal formation form in all probability a part of the septal hippocampal afferents The CA terminals have the same distribution pattern in the hippocampal formation as the degenerated fibres found by CRAGG (1961) after lesions in the medial forebrain bundle

The 5 HT terminals in the nuc suprachiasmaticus show a very marked decrease in number after chronic bilateral lesion of the medial forebrain bundle (ANDEN DAHLSTROM FUXE and LARSSON 1965b) which is known to contain also 5 HT fibre bundles (DAHLSTROM and FUXE 1964a) Since these fibre bundles arise at least mainly from the 5 HT nerve cells of group B7 and B8 it seems probable that the 5 HT terminals in the nuc suprachiasmaticus belong to 5 HT neurons with their cell bodies situated in the nuc raphe dorsalis and medianus

The only area to have a normal content of CA terminals on the ipsilateral side of unilateral lesions involving the crus cerebri and the medial forbrain bundle (ANDEN DAHLSTROM FUXE and LARSSON 1965b) was the

external layer of the median eminence (FUXE 1964) This gives further support to the view that these terminals belong to tubero infundibular neurons (FUXE 1964 DAHLSTROM FUXE OLSON and UNGERSTEDT 1964) There is also other evidence for this view After lesion of the infundibular stalk and median eminence mainly on one side there is a fairly marked increase in the fluorescence intensity in the CA nerve cells of group A12 on this side 2 days after the operation (FUXE HOKFELT and HOKFELT unpublished data) After treatment with reserpine nialamide L DOPA axons with a weak green fluorescence can be traced from the terminals in the external layer up to the region of the nuc arcuatus where the A12 cells are mainly situated

The origins of the monoamine terminals in the lower brain stem are practically unknown It seems highly probable however that they belong to the various CA and 5 HT cell groups present in this part of the brain since monoamine cell bodies have been found nowhere else in the central nervous system

The distribution pattern of the CA terminals is in agreement with the biochemical data (cf BERTLER 1961 ANDEN unpublished data) That of the 5 HT terminals does not so agree since biochemically for instance the hypothalamus and the neostriatum (BERTLER 1961 ANDEN unpublished data) are known to contain fairly high concentrations of 5 HT as compared with the sparse numbers observed with the present method This is in all probability due to the fact that the 5 HT terminals are very fine—many probably sub lightmicroscopical—and sensitive to ultraviolet irradiation In the lower brain stem and the spinal cord however at least most of them seem to be observed

In the spinal cord NA and 5 HT block the short latency transmission from the flexor afferents to the dorsal roots the motoneurons and the ascending tracts (ANDEN JUKES and LUNDBERG 1964) a tetanic stimulation of the flexor reflex afferents therefore evokes after a long latency a depolarization of all the Ia afferents and a discharge of the flexor motoneurons (ANDEN JUKES LUNDBERG and VYKLICKY 1964) [1] 5 HT also excites the motoneurons (ANDEN JUKES and LUNDBERG 1964) [2]

[1] There exists strong support for the view that the block which is obtained after injection of L DOPA or 5 HTP is caused by release of NA and 5 HT from NA and 5 HT terminals These terminals may at least partly descend in the substantia gelatinosa (DAHLSTRÖM and FUXE 1965)

[2] There exists good evidence that the excitation induced in the α motoneurons by stimulation in the caudal medial part of the medulla oblongata is due to the release of 5 HT from 5 HT terminals making close contact with α motoneurons These 5 HT terminals belong to bulbo spinal neurons having their cell bodies in the area stimulated

The 5-HT terminals surrounding the preganglionic sympathetic cells innervating the adrenal medulla may have an inhibitory function (ANDÉN, CARLSSON and HILLARP 1964). Many monoamine terminal systems may have an inhibitory function also in the brain including the retina (MALMFORS 1963; DAHLSTRÖM, FUXE, HILLARP and MALMFORS 1964). It is also possible that the 5-HT terminals in the nuc. salivatorius superior are inhibitory. This would explain the lacrimation-salivation obtained after reserpine (CARLSSON 1965).

Reserpine is also known to interfere with the function of the extrapyramidal motor system (cf. CARLSSON 1965). There is strong support for the view that this is due to a DA depletion of the terminals belonging to the large DA nigro-neostriatal system. More clearcut evidence for this view has recently been obtained by injecting reserpine, haloperidol or chlorpromazine into rats with a chronic unilateral lesion of the fibre bundles in this large DA system. Treatment with these drugs produces head and tail flexion to the side opposite to the lesion together with tremor and flexion of the proximal extremity joints on the side opposite to the lesion (ANDÉN, DAHLSTRÖM, FUXE and LARSSON, to be published).

The presence of a rich plexus of NA terminals in the stratum radiatum of the gyrus hippocampi and in the part just beneath the granular layer of the gyrus dentatus agrees well with the fact that reserpine decreases the threshold for eliciting hippocampal seizure discharges. Furthermore, NA and DOPA administered intracisternally and intravenously respectively, lower this threshold (KIMISHIMA 1963). These results suggest that the NA terminals play an important role in the action of reserpine on hippocampal seizure.

It has recently been shown that 5-HT neuron systems are probably involved in the inhibition of estrous behaviour in rats, the site of action probably being localized in the anterior hypothalamus (MEYERSON 1964). Further support for this view has been obtained by histochemical studies on this area revealing the presence of 5-HT terminals especially in the nuc. suprachiasmaticus. Thus, it may be that the 5-HT terminals in this nucleus are involved in heat inhibition. Experiments are in progress to see if the amine contents of these terminals can be changed by hormone treatment.

The application of minute crystals of A and NA to certain parts of the hypothalamus and the preoptic area (HERNANDEZ-PEÓN, CHAVEZ-IBARRA, MORGANE and TIMO-IARIA 1963) and microinjections of NA in the rat hypothalamus (MILLER, GOTTESMANN and EMERY 1964) have given interesting results and the possibility exists that an excitation or inhibition of normally adrenergically innervated neurons is taking place.

It has recently been shown that it is possible by means of electrical stimulation to obtain a depletion of the monoamine terminals (DAHLSTRÖM, FUXE, KERNELL and SEDVALL 1965, FUXE and GUNNE 1964) if resynthesis is inhibited with the help of the synthesis inhibitors recently developed by CARLSSON, CORRODI *et al* (CARLSSON, CORRODI and WALDECK 1963, CARLSSON and CORRODI 1964). Since this depletion can be directly demonstrated there seems now to be available a quite new method for demonstrating states of activity in the different monoamine systems in the central nervous system. This seems to be an important opening for demonstrating the function of the different monoamine systems. Experiments of this type are now in progress and preliminary results seem to indicate that some system of NA terminals in certain hypothalamic nuclei are in high activity under physiological conditions.

The various CA and 5-HT terminal systems presented the same appearance whether dissection of the brain had occurred *in vivo* or immediately after killing. This does not support the view of so called "stable" and "labile" monoamine stores in the central nervous system as introduced by CSILLIK and ERULKAR (1964). The suggestion made by these authors that the monoamines are present in dendrites can be directly rejected in view of the present results. Their results are in all probability caused by the use of a cryostat technique which in this laboratory has been found inadequate for the study of monoamine terminals in the central nervous system since diffusion of the amines can hardly be avoided.

SUMMARY

With the help of the highly specific and sensitive fluorescence method of FALCK and HILLARP there was studied the distribution of the various systems of synaptic terminals of DA NA and 5 HT neurons in the central nervous system

The DA nerve terminals are present mainly in circumscribed regions in the telecephalon (*i e* in the neostriatum tuberculum olfactorium and nuc accumbens) and in the median eminence NA terminals have a more wide spread distribution and have been found in greater or lesser numbers in the vast majority of the areas of the central nervous system from the neocortex to the spinal cord They form a very rich plexus in *e g* many areas of *the limbic lobe many nuclei of the vegetative hypothalamus many of the* vi ceral afferent and efferent nuclei of the cranial nerves and certain parts of the reticular formation and the raphe complex

The 5 HT terminals are observed mainly in the lower brain stem and the spinal cord the density of the terminals varying considerably between the different nuclei In most parts of the diencephalon and the telencephalon there are usually seen only a very low to low density of 5 HT terminals or none at all probably due to the fineness and ultraviolet instability of these 5 HT terminals

The DA and 5 HT terminals are for the mo t part very fine while the NA terminals are usually somewhat thicker The latter however vary greatly in size in the hypothalamus they are mainly fine to fairly thick while in the hippocampal formation and the neocortex they are almost exclusively very fine

The various CA and 5 HT terminal systems show fairly marked differences in their rates of depletion after reserpine administration and in their sen sitivity to reserpine Differences were observed al o between the terminals of an individual system although these were not so marked The highest depletion rates were found in the areas rich in DA terminals

No obvious differences were observed between the various CA terminal systems in their rates of depletion and recovery after administration of m tyro ine or tetrabenazine nor did the 5-HT terminal systems show any obvious differences in their rates of amine accumulation after MAO in hibition

The CA and 5 HT neurons have a high specificity in respect of the uptake and/or decarboxylation of DOPA and 5 HTP respectively

The origins of the monoamine terminals in the spinal cord in the telencephalon and the diencephalon have to a large extent been established in experiments made in cooperation with Dr A CARLSSON and his research group and Dr K LARSSON (Departments of Pharmacology and Psychology, University of Goteborg) The monoamine terminals in the spinal cord belong at least mainly to several descending bulbospinal NA and 5 HT neuron systems The DA terminals in the neostriatum belong to a large ascending nigro neostriatal neuron system The CA terminals in the limbic lobe (e g the hippocampal formation amygdaloid cortex septal area) the hypothalamus and the preoptic area belong at least mainly to neuron systems *which originate from the mesencephalon (mainly cell group A10)* and to a minor extent probably also from other cell groups in the lower brain stem and which ascend at least mainly in the medial forebrain bundle The 5 HT terminals in the nuc suprachiasmaticus belong in all probability to neuron systems with cell bodies in the mesencephalon (groups B7 and B8) and fibres ascending in the medial forebrain bundle

The function of the various monoamine neuron systems is briefly discussed It has been shown to be possible to study directly the state of activity of the monoamine terminals with the help of synthesis *inhibitors* This has opened a new approach for studies on the function of different terminal systems

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FIG 1 Lateral part of the anterior horn of the cervical intumescence Nialamide treated rat Transverse section There is a high accumulation of NA and 5 HT terminals in the ventro lateral part (*A*) which seems mainly to correspond to the area of the dorsolateral group of the lateral motor cells Medially (*B*) the density is much lower The ventral direction is indicated by the arrow This arrow has the same function also in the other microphotos where it appears × 100

FIG Lateral part of the anterior horn of the cervical enlargement Nialamide treated rat Transverse section A large number of NA and 5 HT terminals is present in the ventrolateral part (*A*) while the dorsomedial part (*B*) contains considerably fewer

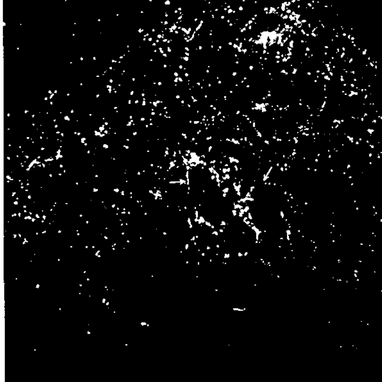


Fig 3 Ventral part of the anterior horn of the spinal cord in the border zone between the thoracic and lumbar spinal cord Normal rat Transverse section The small aggregation of large nerve cells lying closely packed in the central part of the horn is exclusively innervated by 5-HT terminals intimately surrounding the nerve cells (↗) A fairly large number of NA and 5-HT terminals (the 5-HT terminals now at least as frequent as the NA terminals) are present in the rest of the ventral horn especially in the lateral part They seem to lie mainly between the nerve cells × 170

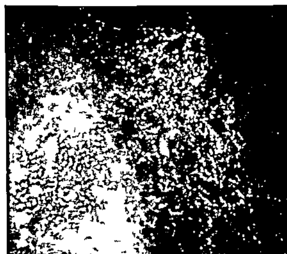


Fig 4 Anterior two thirds of the ventral horn of the caudal part of the thoracic spinal cord (Th 10) Normal rat Transverse section A high density of NA and 5-HT terminals is present everywhere except the most ventromedial part (*A*) × 120

Fig 5 Ventral part of the anterior horn of normal rat Thoracic part Longitudinal section A rich plexus of NA and 5 HT terminals surrounds large nerve cell bodies making intimate contacts (↗) with at least some of them × 1 0

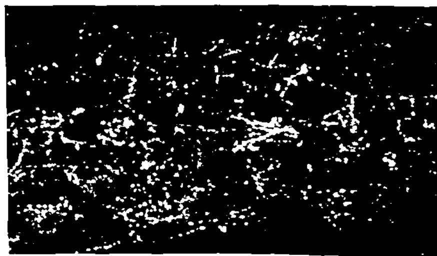


Fig 6 Ventral part of the anterior horn of normal rat Thoracic part Longitudinal section Fine varicose NA and 5 HT terminals make intimate contacts (↗) with numerous nerve cell bodies at least some of which are probably the cell bodies of α motoneurons × 280

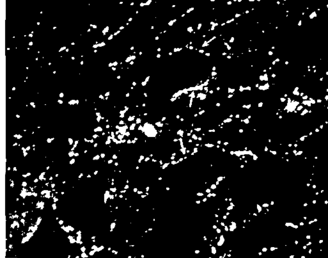




Fig 7 Ventral part of the anterior horn of normal rat Thoracic part Longitudinal section A large number of NA and 5 HT terminals make intimate contacts (↗) with large nerve cell bodies 280

Fig 8 Ventral part of the anterior horn of normal rat Thoracic part Longitudinal section NA terminals make intimate contacts with a small nerve cell body 460



Fig 9 Ventral part of the anterior horn of normal rat Thoracic part Longitudinal section 5 HT terminals make intimate contacts with nerve cell bodies of different sizes ( ) 210

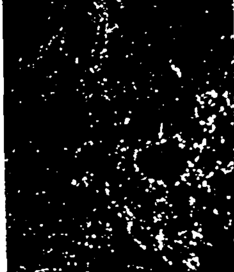


Fig 10 Ventral part of the anterior horn of normal rat Thoracic part Longitudinal section NA terminals are observed to make intimate contacts not only with large nerve cell bodies (*) but also with processes (↗) × 280

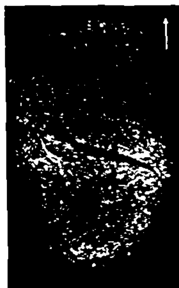


Fig 11 Posterior part of the dorsal horn of normal rat Thoracic part Transverse section A high accumulation of descending or ascending NA terminals is present in the part (A) which seems at least mainly to correspond to the substantia gelatinosa They have been transversely cut and appear as strongly green fluorescent dots in the fluorescence microscope × 80

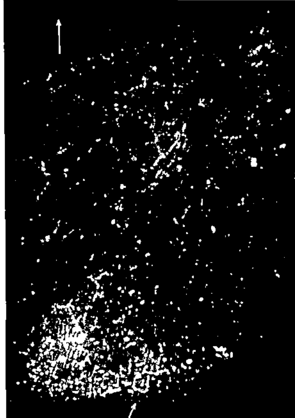


FIG. 1. Dorsal horn of the cervical spinal cord of normal rat. Transverse section. In the area which seems to correspond to the substantia gelatinosa (*A*) there is a high accumulation of descending or ascending NA terminals which have been transversely transected and appear in the fluorescence microscope as strongly green fluorescent dots. The rest of the dorsal horn contains a much lesser amount of NA terminals (*B*). The posterior surface of the spinal cord is indicated (↗). ×130

FIGS. 13 and 14. Substantia gelatinosa of the spinal cord of normal rat. Longitudinal section. Fine NA terminals descend or ascend in this zone running fairly parallel with each other with little or no branching and sometimes making intimate contacts with the small cell bodies of the interneurons (Fig. 13 ↗). Mainly, however, they run between the cell bodies and probably make intimate contacts with the rich dendrite net which is oriented in the same direction as the terminals. ×280





FIG 15 Sympathetic lateral column of normal rat Thoracic region Longitudinal section Since this sympathetic cell column has a beaded appearance the NA and 5 HT terminals will not form a continuous and densely packed column but a discontinuous column (↙) The terminal are highly accumulated only in areas where the sympathetic cell bodies and processes are to be found Monoamine fibres (↗) are observed to enter the nucleus from the lateral funiculus (left) × 40

FIG 16 Sympathetic lateral column of the upper thoracic segments Normal rat Longitudinal section A very high density of mainly NA terminals surrounding the preganglionic sympathetic nerve cells The lateral funiculus is present to the left of the figure × 170

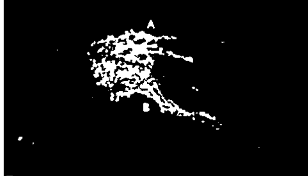


Fig 17 Sympathetic lateral column of a nialamide treated rat Thoracic part Transverse section The NA terminals form a dense plexus mainly in the ventral part (A) and the 5 HT terminals in the dorsal part (B) 170

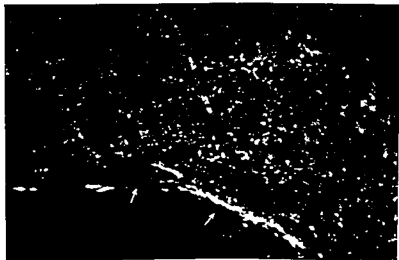


Fig 18 Ventral surface of the brain at the border between the medulla oblongata and the spinal cord of a nialamide treated rat Transverse section Due to MAO inhibition the smooth 5 HT axons which lie just under the ventral surface (↗) laterally of the pyramidal tract have developed a fairly strong yellow fluorescence They have been transversely cut and appear as fine dots 10



Fig. 19. Medial surface of the anterior funiculus of a reserpine-nialamide-treated rat. Transverse section. Due to MAO inhibition the smooth 5-HT axons have developed a fairly strong yellow fluorescence and outline the medial surface of the funiculus as a rim of yellow fluorescent dots (↗), which represent the transversely cut axons. ×170

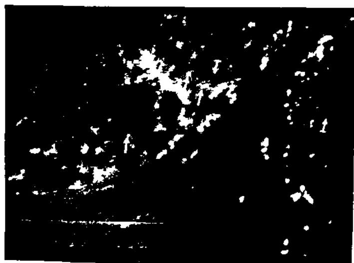


Fig. 20. Spinal cord at meeting of the ventral and medial curvature of the anterior funiculus. Nialamide-treated rat. Transverse section. The smooth 5-HT axons have developed a fairly strong yellow fluorescence and appear as fine dots (↗). These are not surrounded by weakly brownish fluorescent myelin rings (↗) and thus appear to be unmyelinated. ×460

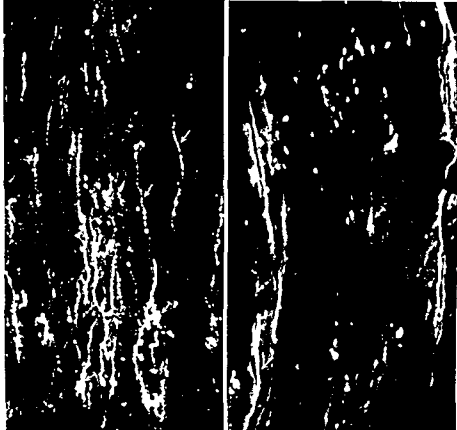


FIG _1 Anterior funiculu ju t above a _ day old total tran ection of the spinal cord (C8) Longitudinal secti n A fairly large number of deformed NA and some deformed 5 HT fibres are ob erved with an intense green and yellow flu rescence inten ity re pectively Both beaded (↗) and convoluted (↗) configurations are ob rved The bottom of the figure is cl e to the le ion 1"0

FIG _ Lateral funiculus above a 2 day old total tran ection of th pinal cord (C8) Nialamide treated rat Longitudinal sect on The smooth 5 HT fibres (↗) have devel oped the strong yellow fluorescence that always occurs after MAO inhibition The strongly green fluore cent dot (↗) represent the enlargements of deformed NA fibres with a con id rably increa ed amine content present about 4 mm abo e the lesion 280

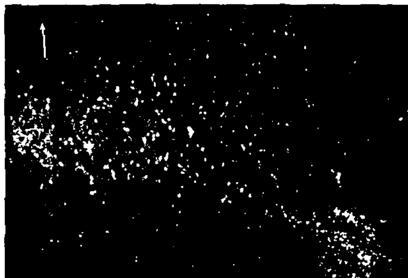


Fig. 23 Border zone between the anterior and lateral funiculus above a 2 day old total transection of the spinal cord at Th 4. Transverse section. The medial direction in the figure is to the right. The tip of the ventral horn is present at A. A large number of NA fibres are observed with a fairly strong green fluorescence intensity due to an accumulation of CA. × 130



Fig. 24 Anterior funiculus and the most ventral part of the lateral funiculus just above a 3 day old total transection of the rat spinal cord at Th 4. Transverse section. A large number of fibers with a strong green fluorescence intensity due to an accumulation of NA are observed within the anterior funiculus (*A*) and the most ventral part of the lateral funiculus (*B*). A number of 5-HT fibres with a strong yellow fluorescence intensity due to an accumulation of 5-HT are found in a very superficial position close to the ventral ( ) and especially the medial (↗) surface of the anterior funiculus. × 30



Fig. 25 Lateral funiculus (mainly the dorsal part) just above a 2-day-old total transection of the spinal cord Transverse section A large number of deformed 5 HT fibres with a strong yellow fluorescence intensity due to an accumulation of 5 HT is found in the dorsolateral part (A) Some such fibres are found also in the area marked B which lies superficially in the middle third of the lateral funiculus A fairly large number of NA fibres with a strong green fluorescence due to an accumulation of NA are found within the area C in the dorsolateral part The sympathetic lateral column is present at D and the superficial part of the dorsal horn at E × 100

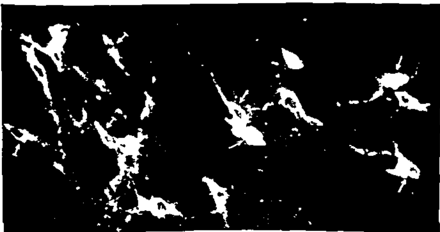


Fig. 26 Group A1 (see Dahlström and Fuxe 1964a) of a rat with 2 day old total transection of the spinal cord (C8) Transverse section through the medulla oblongata Most of the A1 cells have increased their CA content considerably and show a strong fluorescence intensity (↗) They also appear to be swollen and larger than usual Some cell bodies (↗) show no increase in fluorescence intensity × 80





FIG 27a Group A2 of rat with a 2 day old total transection at C8 Transverse section through the medulla oblongata One of the cells (↗) has a very strong green fluorescence intensity in the cell body but not in the process (✓) while the other cell body exhibits a weak green fluorescence intensity only The large numbers of CA terminals present in this area do not appear in the picture ×460

FIG 27b Group A1 of a rat with a 2 day old unilateral transection at C8 Transverse section through the medulla oblongata One CA cell body (↗) with a considerably increased amine content and one (✓) with a normal amine content are observed as in fig 27a ×280

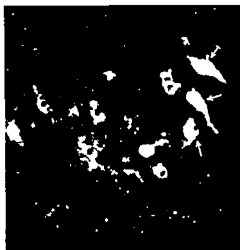


FIG 28 GROUP A2 of a rat with a 2 day old total transection of the spinal cord at C8 Transverse section through the medulla oblongata Some nerve cells (↗) with a considerably increased CA content are observed These cells also appear swollen and are larger than usual and one of them (✓) is observed to have an eccentric nucleus The majority of the cells however have a normal appearance The abundant CA terminals in this area do not appear in the microphoto ×170

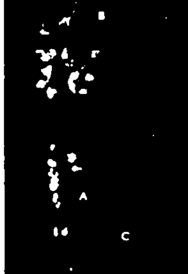


FIG 29 Groups B1 and B2 Reserpine nialamid treated rat Transverse section through the medulla oblongata The small 5 HT cells of group B1 are present mainly within the nuc raphe pallidus (A) and those of group B2 mainly within the nuc raphe obscurus (B) Tractus pyramidalis is present at C ×250



FIG 30 Group B1 and B3 Reserpine nialamid treated rat Transverse section through the medulla oblongata The small 5 HT cells of group B1 are present mainly within the nuc raphe pallidus (A) The medium sized 5 HT cell bodies of group B3 seem to be localized mainly within the part of the reticular formation (B) surrounding the pyramidal tract (C) and in the nuc raphe magnus (D) The cells of group B3 have a strong yellow fluorescence intensity not only in their cell bodies (↗) but also in their processes (↗) and appear to be multipolar

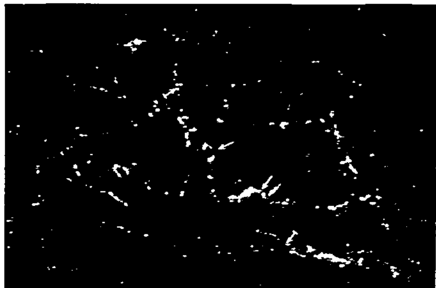


FIG 31 Group B3 of normal rat Transverse section through the medulla oblongata 5 HT nerve cells (→) of weak yellow fluorescence intensity are present in the part of the reticular formation just dorsal to the pyramidal tract A number of fairly thick CA terminals are present (↗) 210

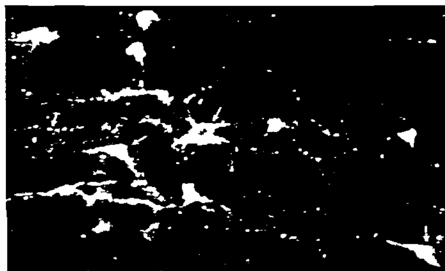


FIG 32 Group B3 of nialamide-treated rat Transverse section through the medulla oblongata 5-HT nerve cells (→) which have developed a strong yellow fluorescence intensity are present in the same area as the 5-HT nerve cells in fig 31 Fairly thick CA terminals are observed (↗) [illegible]

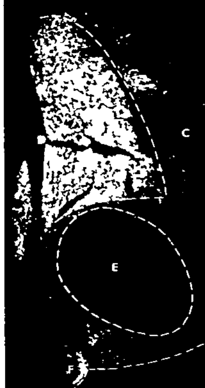


Fig. 33 Transverse section through the vegetative hypothalamus at the level of the posterior part of the median eminence. The transverse sections through the pons and the medulla oblongata were made as perpendicular as possible to the longitudinal axis of the lower brain stem, while those through the mesencephalon, diencephalon and telencephalon were made unless otherwise stated following the axis in the book of KÖNIG and KLIPPEL (1963). Normal rat. The high to very high accumulation of CA terminals present in the dorsal (*A*) and ventral (*B*) part of the nuc. dorsomedialis hypothalami gives a strong green fluorescence intensity to these areas lying mainly between the fornix (*C*) and the third ventricle (*D*). The nu. ventromedialis hypothalami (*E*), on the other hand, which contains only a very low to low density of CA terminals, is practically empty of fluorescent material. The lateral part of the median eminence (*F*) is present in the picture. The external layer shows a strong diffuse green fluorescence intensity due to the presence of densely packed very fine CA terminals. × 40

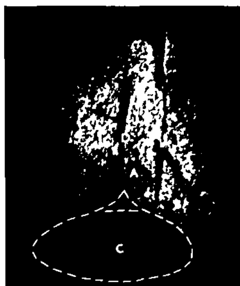


Fig. 34 Preoptic area of normal rat. Transverse section. A high density of CA terminals gives a strong fluorescence intensity to the nuc. preopticus periventricularis (*A*) and to the nuc. preopticus suprachiasmaticus (*B*), both situated dorsal to the chiasma opticum (*C*) and close to the third ventricle (*D*). The medial parts of the nuc. preopticus medialis (*E*) also show a strong fluorescence intensity. ×30

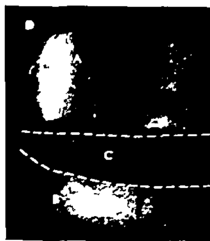




Fig. 35 Nuc. interstitialis striae terminalis of normal rat. Transverse section. The densely packed very fine CA terminals in the dorsolateral part (*A*) just medial to the [illegible] striatum (*D*) and the densely packed fairly thick CA terminals in the ventral part (*B*) close to the commissura anterior (*C*) give a very strong fluorescence intensity to these areas. ×30

Fig. 36 Nuc. anterior ventralis thalami of normal rat. Transverse section. The high density of very fine CA terminals gives a strong fluorescence intensity to this nucleus (*A*). The adjacent nuc. anterior dorsalis thalami (*B*) contains far fewer CA terminals and is thus more or less empty of fluorescent material. ×40



Fig 37 Ventral part of the nuc interstitialis striae terminalis close to the commissura anterior (A) The very high density of CA terminals present gives a strong fluorescence intensity to this nucleus × 80



Fig 38 Retrochiasmatic area of normal rat Transverse section The large to very large number of fine to thick CA terminals give a strong fluorescence intensity to *inter alia* the ventral supraoptic commissure (↗) × 40



Fig 39 Medial part of the tuberculum olfactorium of normal rat Transverse section The insulae calleja (A) are observed to be completely free of fluorescent material while the very fine densely packed DA terminals give a strong diffuse fluorescence intensity to other parts of the tuberculum olfactorium (*D*) The chiasma opticum (*B*) is observed medial to the tuberculum olfactorium and a small artery with its adrenergic plexus close to the outer muscular layer is present at C × 80

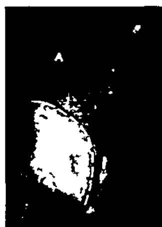




Fig. 40. Dorsal part of the nuc. accumbens of normal rat. Transverse section. The densely packed very fine CA terminals give a strong diffuse fluorescence intensity to the nucleus (*B*). The terminals in the nuc. septalis lateralis (*A*) do not appear in the microphoto. The lateral ventricle is present to the upper left of the figure. × 30

Fig. 41. Nuc. periventricularis hypothalami at the level of the nuc. paraventricularis. Normal rat. Transverse section. The high density of CA terminals in this nucleus (*C*) lying close to the third ventricle (*A*) gives it a strong fluorescence intensity. The adjacent nuc. anterior hypothalami (*B*) has a very low to low density of CA terminals and appears to be practically empty of fluorescence. × 40

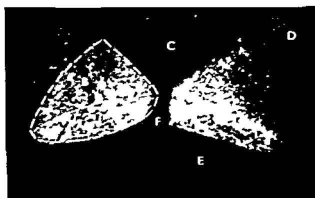


Fig. 42. Nuc. motorius dorsalis n. vagi (*A*) and nuc. tr. solitarii (*B*) of normal rat. Transverse section. The large numbers of CA terminals present usually give a strong fluorescence intensity to these areas. Due to diffusion, however, only a medium fluorescence intensity is observed. The CA terminals in the area postrema (*C*), nuc. gracilis (*D*) and nuc. hypoglossus (*E*) do not appear in the microphoto. The central canal is present at F. × 40

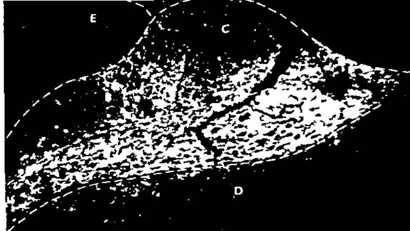


FIG 43 Nuc motorius dorsalis n vagi (*A*) and nuc commissuralis (*B*) of normal rat Sagittal section The very high density of CA terminals in these nuclei gives them a strong fluorescence intensity Some CA terminals are observed also in the area postrema (*C*) and in the nuc hypoglossus (*D*) The nuc gracilis (*E*) is completely free of fluorescence ×80



FIG 44 Part of the reticular formation surrounding the pedunculus cerebellaris superior (*A*) Normal rat Transverse section A high density of CA terminals gives a strong fluorescence intensity to the area of the reticular formation close to the peduncle corresponding mainly to the caudal part of the cuneiform area (*B* medially) and to the area of the nuc lemnisci lateralis (*C* laterally) The cranial part of group A6 (see DAHLSTRÖM and FUXE 1964) is present at D and group A7 (see DAHLSTRÖM and FUXE 1964) at E ×40



Fig. 4e. Nuc. posterior hypothalami of normal rat. Transverse section. A high density of CA terminals is present in the ventral part (*A*) and some medium sized CA nerve cells (A 11) of fairly strong green fluorescence intensity are to be seen in the dorsal part (*B*) medial to the fasciculus mammillotegmentalis (*C*). × 80

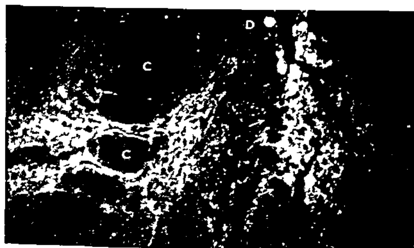


Fig. 4f. Lateral dorsal part of the cranial pons of normal rat. Transverse section made approximately following the B B1 axis of ZEMAN and INNES (1963). A high density of CA terminals give a strong fluorescence intensity to the area (*A*) medial to the nuc. tr. mesencephalici n. trigemini ( ) and which seems to belong to substantia grisea periventricularis and to area (*B*) between certain fasciculi (*C*) of the pedunculus cerebellaris superior. The cranial part of group A6 is observed at D. × 100

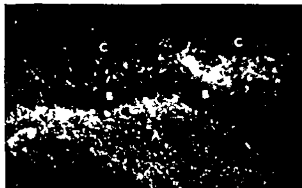


Fig 47 Gyrus dentatus of normal rat Transverse section A high density of CA terminals is present within the layer (A) beneath the stratum granulare (B) which contains only a few CA terminals The stratum moleculare (C) has a very low to low density of CA terminals mainly oriented perpendicular to the outer surface of the gyrus ×80

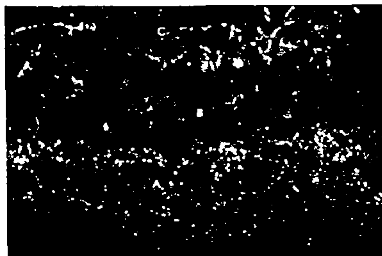


Fig 48 Gyrus dentatus of normal rat Transverse section A high density of very fine CA terminals is present in the layer (A) beneath the stratum granulare (B) which contains only a few CA terminals The stratum moleculare (C) contains only a very low to low density of CA terminals The rich plexus of CA terminals present in layer A seems only occasionally to make contact with cell bodies ×210

Fig 49 Ventral part of nuc dorsomedialis hypothalami of normal cat Transverse section A high density of CA terminals is present The third ventricle is seen to the left (A) × 100

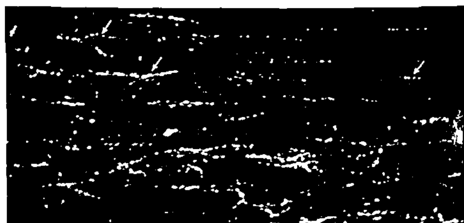


Fig 50 Ventral supraoptic commissure of normal rat Transverse section A large number of fine to fairly thick CA terminals (↗) are present probably making parallel contacts with the dendrites in this area which are oriented in the same manner × 1[illegible]0

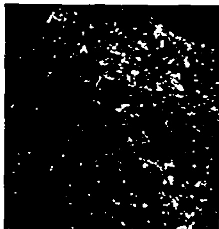


Fig 4 Gyrus cinguli of normal rat Transverse section A medium density of CA terminals is present in the surface layer (A) while the adjacent layers have only a very low to low density × 130

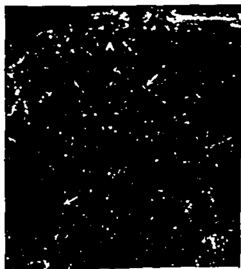


Fig 5 Medial part of the gyrus parietale of normal rat Transverse section The lamina zonalis (*A*) has a low density of CA terminals running mainly parallel to the surface of the brain while the adjacent layers have a very low density of CA terminals which have a fairly irregular course but are mainly oriented perpendicular to the surface and not parallel to it The terminals (↗) lie mostly between the nerve cell bodies × 130

Fig. 3 Ventral part of the griseum centralis of normal rat Transverse section A dense accumulation of mainly fine to fairly thick CA terminals is present in the ventrolateral part (to the left of the figure) while a much less dense meshwork of CA terminals is observed in the ventromedial part (to the right of the figure) ×280

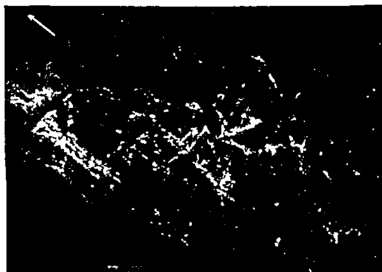


Fig. 4 Mesencephalic reticular formation of normal cat Transverse section A large number of fine CA terminals are present in an area (*A*) present lateral to the dorsal part of the cranial raphe region ×80



FIGS 5 and 6 Parts of the reticular formation which seems to belong to the dorsal and ventral part of the subnucleus reticularis ventralis medullae oblongatae respectively Normal rat A low to medium density of CA terminals is observed and also a very low density of 5 HT terminals The terminals make intimate contacts with some of the reticular nerve cell bodies (↗) and their processes (↗) ×170

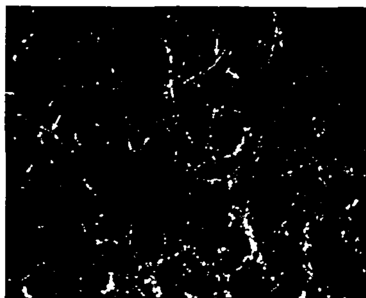


Fig. 57 The ventromedial part of the reticular formation of the medulla oblongata overlying the pyramidal tract just cranial to the inferior olivary complex. Normal rat. Transverse section. A low density of fairly thick CA terminals (↗) of strong green fluorescence intensity is present, as are a small number of very fine 5 HT terminals of weak to medium yellow fluorescence intensity (↗) ×170

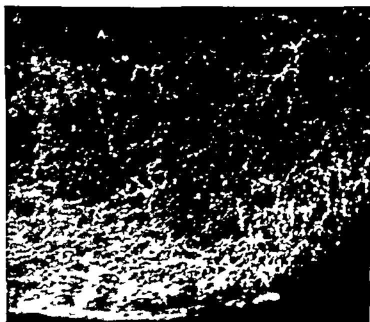


Fig. 8 The part of the hypothalamus ventral to the recessus mammillare and partly within nucleus arcuatus at the level of the cranial part of the corpus mammillare. Normal cat. Transverse section. A high density of fine to fairly thick CA terminals is present just below the surface of the hypothalamus, a much less dense plexus being present in the rest of the area. The ventral surface of the recessus mammillare is present at A

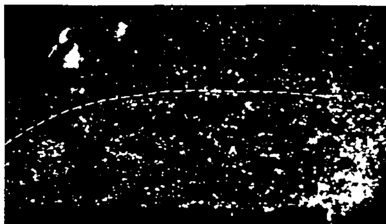


Fig. 59. Nuc. motorius dorsalis n. vagi (A) of normal rat. Transverse section. A high density of fine CA terminals is present between the nerve cells of the nucleus, especially in the part close to the central canal (*B*). CA nerve cells (↗) belonging to group A2 (see DAHLSTRÖM and FUXE 1964) are present within the nuc. commissuralis. × 210

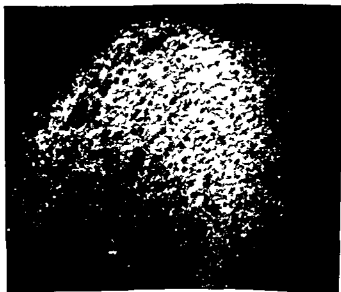


Fig. 60. Nuc. anterior ventralis thalami of normal rat. Transverse section. A dense meshwork of very fine CA terminals fill up the entire nucleus. × 1[illegible]0

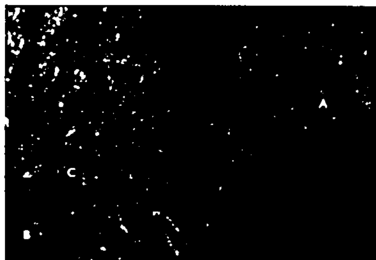


Fig. 61 Posterior part of the nuc. arcuatus of normal rat. Transverse section. A medium density of mainly fine CA terminals is present within the nucleus (*C*). The adjacent nuc. ventromedialis hypothalami (*A*) contains far fewer CA terminals. The ventral part of the third ventricle is seen at B. The A12 cells (see Fuxe 1964; Dahlström and Fuxe 1964*a*) are present mainly in the anterior part of the nuc. arcuatus. × 130

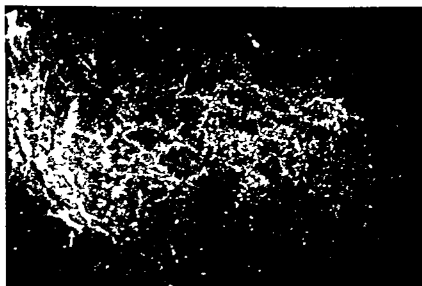


Fig. 62 Nuc. motorius n. trigemini of normal rat. Transverse section. A high density of CA terminals is present intimately surrounding the motor nerve cell bodies (*A*). Some of the numerous 5-HT terminals also appear in the picture. Some medium sized CA nerve cells of strong green fluorescence intensity are observed medial to the nucleus (↗). These are continuous with the ventral tip of the locus coeruleus. × 100

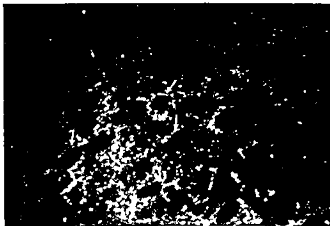


FIG 63 Ventral caudal part of the nuc hypoglossus of normal rat Transverse section This part contains a high den ity of CA terminals which intimately urround the motor nerve cell bodies (↗) 210

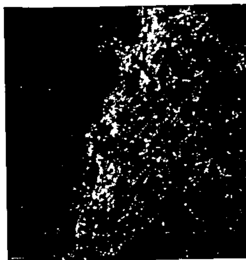




FIG 64 Substantia gelatinosa (A) of the medulla oblongata of normal rat Transverse section A low den ity of fine to very fine CA terminals is observed The densely packed very f ne 5 HT terminals present in this area do not appear in the microphoto To the r ght of the f gure is the nuc t spinalis n trigemini with a very low den ity of CA terminals 1 0

FIG 65 Sub tantia gelatinosa of the medulla oblongata of a nialamide treated rat Transverse sect on The 5 HT terminals appear as densely packed yellow fluorescent dot (A) Owing to a certain degree of diffusion the CA terminals are absent or appear only indistinctly 80

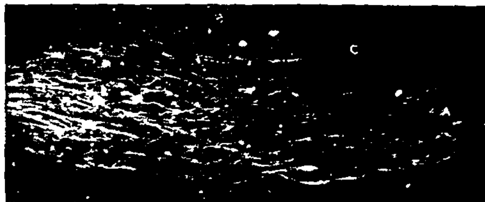


Fig. 66. The midline border zone between the ventral part of the mesencephalon and the hypothalamus. Reserpine-nialamide treated rat. Transverse section cut mainly according to the B-B1 axis in the book of ZEMAN and INNES (1963). A tract of 5-HT fibre bundles with a fairly strong yellow fluorescence is observed to pass from the mesencephalon (A) into the median forebrain bundle (B) medial to the fasciculus retroflexus (C). ×100



Fig. 67. Nuc. dorsalis corporis geniculati lateralis of normal rat. Transverse section. A low density of very fine CA terminals is observed. Most of the terminals lie between the cell bodies but some of them make axosomatic contacts (↗). ×280

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The use of rat and mouse iris for direct observations on their physiology and pharmacology at cellular and subcellular levels

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KEY TO THE TABLES

Numbers in parentheses

( ) – Total number of animals

( )* Animals treated with reserpine (10 mg/kg i p)
4 h before administration of CA

([illegible]) Animals treated with reserpine (1 mg/kg)
24 h before administration of CA

[illegible] *Average fluorescence of the adrenergic ground plexus*

[illegible] Very strong

– – strong

– moderate

weak

none

[illegible] kg i p [illegible] h before the administration of CA or L DOPA unless otherwise stated

[illegible] kg [illegible] p [illegible] 18 (15 to 21) h before the administration of CA or L-DOPA unless otherwise stated

INTRODUCTION

The mechanisms for the uptake storage and release of CA[1] especially the transmitter NA in adrenergic nerves and the effects of drugs on these mechanisms have been extensively studied by means of modern biochemical techniques (for reference see AXELROD 1964 COSTA and BRODIE 1964 STJÄRNE 1964 CARLSSON 1965*a*) It has been shown for instance that the nerves in all probability can take up and store NA and closely related amines (AXELROD WEIL MALHERBE and TOMCHICK 1959 MUSCHOLL 1960 HERTTING and AXELROD 1961 HERTTING AXELROD KOPIN and WHITBY 1961 STROMBLAD and NICKERSON 1961 WHITBY AXELROD and WEIL MALHERBE 1961 ANDÉN CARLSSON and WALDECK 1963) This has been further supported by autoradiographic studies (MARKS SAMORAJSKI and WEBSTER 1962 WOLFE POTTER RICHARDSON and AXELROD 1962) The new biochemical techniques have greatly improved our opportunities of studying the adrenergic transmission

However the results obtained in such experiments are sometimes difficult to interpret It has been impossible for instance with the chemical methods to obtain any exact knowledge as to what parts of the nerves the amines are taken up in and stored or where the uptake and concentration mechanisms are localized The lack of this important information is one of the reasons why views differ so markedly as regards the site and mode of action of many drugs (e g reserpine) of great interest in the pharmacology of the adrenergic nervous system

It has been shown however that the amine granules in both the adrenal medulla (CARLSSON HILLARP and WALDECK 1962 1963 KIRSHNER 1962 *a* and *b*) and the adrenergic nerves (EULER and LISHAJKO 1961*a* *b* 1963*a* *b* and *c* STJÄRNE 1964) have specific uptake storage mechanisms which are blocked by certain drugs e g reserpine On the basis of certain experiments made on intact amine storing cells CARLSSON HILLARP and WALDECK (1963) have

[1] *Abbreviations used* NA = noradrenaline H³NA = tritium labelled noradrenaline DA = dopamine CA = catecholamine(s) DOPA = 3 4 dihydroxyphenylalanine DOPS = 3 4 dihydroxyphenylserine 5 HT = 5 hydroxytryptamine 5 HTP = 5 hydroxytryptophan MAO = monoamine oxidase MAOI = monoamine oxidase inhibitor(s) COMT = catechol O methyl transferase

MATERIAL AND METHODS

ANIMALS About 3000 adult predominantly female albino rats (Sprague Dawley weight approximately 200 g) about 100 adult albino mice of both sexes (weight approximately 30 g) and 4 adult albino guinea pigs (weight approximately 300 g) were used

HISTOCHEMICAL PROCEDURES Unless otherwise stated, the animals were killed by decapitation under light ether anaesthesia The eyes were in most cases removed and dissected immediately In some cases the eyes were left *in situ* 15 to 60 min post mortem

The bulbs were divided 1 to 2 mm behind the corneo scleral junction with a pair of razor blades and the anterior half placed on a glass slide with the cornea downwards under a Zeiss operation micro cope or a Zeiss stereomicroscope (magnification 6 to 10 ×) The lens was removed with its capsule which appeared to be fixed to the remaining anterior part of the retina The ciliary body easily recognizable from its abundance of blood vessels was then together with the anterior parts of the choroid and the iris easily dissected free from the sclera with a small special lancet and fine tweezers The iris—with the anterior surface usually upwards—and the ciliary body were cut radially and drawn to a dry place on the slide where the pupil edge was first stretched and allowed to dry for about 15 to 30 sec and adhere to the slide The rest of the iris and the ciliary body with fragments of the choroid were then stretched and allowed to dry into place for 30 to 60 sec

Immediately after preparation and within about 5 min from the time dissection started the slides were placed in a metal holder for 24 slides in a desiccator containing phosphorous pentoxide (Sicapent Th Schuchardt GmbH Munich) for further drying for at least 1 h They could be stored in this for up to 24 h before formaldehyde treatment

The preparations from one metal holder were transferred to a glass vessel (1 l) closed by a tightly fitting lid with a packing medium (vacuum grease or rubber ring and metal chip) and containing a layer of paraformaldehyde powder (6 g) on the bottom These vessels were kept at +80 C for 1 h The paraformaldehyde (purum E Merck Darmstadt Germany)

was standardized in respect of adsorbed water content by keeping it in closed vessels of a constant relative humidity (10 to 90 per cent) produced by different concentrations of sulphuric acid In this way the paraformaldehyde could be standardized to give at +80 C a formaldehyde gas with a water content sufficiently high for an optimal reaction i e transformation of primary CA to intensely fluorescent 6 7 dihydroxy 3 4 dihydroisoquinolines (CORRODI and HILLARP 1963 1964) but low enough to avoid diffusion (for details see HAMBERGER MALMFORS and SACHS 1965) The paraformaldehyde was tested with the use of normal irises which were treated immediately before the preparations from the experimental animals To get a further check that the reaction in each vessel was satisfactory normal irises were always treated together with the experimental material in the same vessel After formaldehyde treatment the preparations could be kept in a dark dry place for at least one week without any obvious change in specific fluorescence

For comparison the anterior halves of eyes from normal and experimental animals were either immediately or 15 min post mortem rapidly frozen in propene/propane (4 1) cooled with liquid nitrogen They were then dried *in vacuo* for 5 to 6 days treated with formaldehyde gas of optimum water content (see above) at +80 C for 1 h embedded in paraffin sectioned at 5 to 10 μ and mounted in Entellan (Merck) for fluorescence microscopy The entire procedure which follows in the main that originally developed by FALCK (1962) has recently been described in detail by DAHLSTRÖM and FUXE (1964*a*)

The whole mounts of irises—unmounted or mounted in Entellan—and the sections of the freeze dried specimens were examined with a Zeiss monocular fluorescence microscope fitted with a high pressure mercury lamp HBO 200 (Osram) and BG 12 filters (3 to 4 mm Schott & Gen Germany) for activation and 2 KG 1 filters (1 mm Schott & Gen Germany) for heat absorption The stop filter in the tube was a Zeiss 50 filter (2 mm) which corresponds roughly to an OG 4 filter (3 mm Schott & Gen Germany) A dark field condenser for oil immersion (Zeiss) was used throughout The lenses were planeachromates and non fluorescent Gevaert Scopix G 35 mm film was used for the microphotographs Exposure times varied between 30 and 120 sec The iris preparations were mounted before exposure in pure Entellan with or without a cover glass to avoid diffraction

ADMINISTRATION OF AMINES AND OTHER AGENTS Intravenous and intraocular injections in the rat were made under ether pentobarbital sodium

(30 to 35 mg/kg i p, reserpinized animals only 20 to 25 mg/kg) or urethane (1 g/kg s c or i p) anaesthesia

The intravenous administrations of CA in rat were made in the lingual vein (ANDERSON 1963) usually by a slow (1 to 2 min) but sometimes by a rapid (6 sec) injection of 0 2 ml or by a continuous infusion of 1 ml during 10 to 50 min In mouse the intravenous injections were made during 2 min (0 1 ml) in one of the tail veins

Intraocular injections in the anterior chamber were made in rat only under a Zeiss operation microscope (magnification 16 ×) using a microlitre syringe with a very fine needle (H 705 Hamilton Company needle no 22) The sclera was penetrated 1 to 2 mm behind the corneo scleral junction The point of the needle was inserted carefully into the anterior chamber of the eye through the pupil having passed through the outer gelatinous zone and the capsule of the lens It is important to avoid bleeding from the iris and penetration of the cornea and to make sure that the point of the needle has really penetrated the capsule of the lens A volume of aqueous humor roughly corresponding to that of the solution to be injected was first sucked out with one syringe which then was withdrawn with its needle Another syringe with the same type of needle was then inserted in the previous hole and the solution injected into the anterior chamber The usual injection was 10 μl which was calculated to give an initial concentration in the anterior chamber which was about half that of the administered solution (see chapter II) One of the eyes usually received the solvent alone and served as a control

DRUGS ADMINISTERED a Reserpine (Serpasil® Ciba 0 1 to 10 mg/kg) was administered i p or i v 0 5 h to 8 days before preparation Dilutions were performed when necessary with the solvent or 5 5 per cent glucose Reserpine was also dissolved on occasion in a minimal amount of 10 N acetic acid and diluted after the addition of a few drops of absolute ethyl alcohol with 5 5 per cent glucose For local administration in the anterior chamber of the eye reserpine phosphate (0 01 to 1 mg/ml) was dissolved in 5 5 per cent glucose

b The following MAOI were dissolved in 0 9 per cent sodium chloride and given at the stated intervals before the administration of CA or death (the doses refer to the salts)

pargyline hydrochloride (MO 911 Eutonyl® Abbott Lab N methyl N benzyl 2 propynylamine 200 mg/kg i p 4 to 6 h)

pheniprazine (JB 516 Catran® Draco β phenylisopropylhydrazine 10 mg/kg i v 4 h)

iproniazid phosphate (Marsilid[R] Hoffman La Roche N isopropyl N iso nicotinyl hydrazine 100 mg/kg i v 12 to 17 h)
harmaline hydrochloride (3 4 dihydro 7 methoxy 1 methyl 9 pyrid /3 4 b/ indole 20 mg/kg i p 0 5 h)
harmine hydrochloride (7 methoxy 1 methyl 9 pyrid /3 4 b/ indole 20 mg/kg i p 0 5 h or 2 mg/kg i v 0 25 h)

Nialamide (Niamid[R] Pfizer N i onicotinoyl N /β (N benzyl carboxy amido)ethyl/hydrazine 5 to 500 mg/kg) was dissolved in 0 9 per cent sodium chloride with the help of a minimal amount of 3 N HCl The solution was then carefully brought to approximately pH 5 with N NaOH and injected i p 0 5 to 24 h before the administration of CA or death

Only freshly made solutions of the hydrazines were used

c The laevo and dextro forms of NA (bitartrate and hydrochloride respectively) and the laevo form of α methyl NA (hydrochloride Corbasil[R] Hoechst) were administered i v (0 01 to 0 5 mg/kg) s c or i p (0 5 to 5 mg/kg) *For the intraocular injections in the anterior chamber* 2×10^{-9} *to* 2×10^{-4} *M* solutions were used giving initial concentrations in the anterior chamber of about 10^{-9} to 10^{-4} *M* (0 0035 ng to 0 35 μg/eye)

DA hydrochloride and α methyl DA hydrochloride were administered i v (0 05 to 5 mg/kg) or i p (2 to 10 mg/kg) For intraocular injections in the anterior chamber 2×10^{-7} to 2×10^{-5} *M* solutions were used

CA was administered 0 1 to 8 h before preparation All the figures given are amounts or concentrations of the amines calculated as free base The amines were dissolved in 0 9 per cent sodium chloride The solutions containing 0 1 mg/ml or less were freshly prepared on each occasion by the dilution of a stock solution (1 mg/ml in 0 001 N HCl) kept at −20 C Solutions containing less than 10 μg/ml were made by dilution with a freshly made solution (about pH 6) containing sodium chloride (0 9 per cent) ascorbic acid (20 μg/ml) and EDTA (20 μg/ml) and kept at 0 C for not longer than 4 h to avoid destruction of the CA by oxidation Sodium EDTA was obtained from a stock solution (10 mg/ml pH 6) stored at 0 C

5 HT creatinine sulphate was dissolved in 0 9 per cent sodium chloride and administered i p (20 to 100 mg/kg free base) 1 h before death

DL 5 HTP (100 mg/kg i p) was dissolved in 0 9 per cent sodium chloride and administered 1 h before death

L and D DOPA were dissolved in 0 9 per cent sodium chloride with the help of a minimal amount of N HCl and gentle warming Polyethyleneglycol (0 02 ml/ml) was then added to the warm solution and the pH carefully brought to about 5 with N NaOH The solutions were then immediately

injected i v (0 2 to 5 mg/kg) or i p (1 to 20 mg/kg) 0 1 to 1 h before death Specially composed solutions (2×10^{-7} to 2×10^{-4} *M* in phosphate buffer) were used for most of the intraocular administrations

α methyl DOPA (Aldomet® Merck Sharp & Dohme) the laevo form and DL-threo DOPS were dissolved in 0 9 per cent sodium chloride and given i p (10 to 50 mg/kg) or s c (10 mg/kg) 1 h before death

d NSD 1015 (3 hydroxybenzyl hydrazine fumarate Smith and Nephew Research Ltd) was dissolved in 0 9 per cent sodium chloride given i p (100 mg/kg) 1 to 4 h before administration of any of the above mentioned amino acids

e The following drugs were dissolved in 0 9 per cent sodium chloride (unless otherwise stated they are hydrochlorides and the doses refer to the salts)

tyramine (2 to 50 mg/kg i p)
(−) metaraminol bitartrate (Aramine® Merck Sharp & Dohme 10 mg/kg —free base—i p)
(+) amphetamine sulphate (10 mg/kg i p)
bretylium tosylate (50 mg/kg s c or 10 to 50 mg/kg i p)
cocaine (20 mg/kg i p)
guanethidine sulfate (Ismelin® Ciba 10 to 30 mg/kg i p)
ouabain (g strophanthin 10 mg/kg i v)
phenoxybenzamine (10 mg/kg i v or 50 mg/kg i p)
imipramine (Tofranil® Geigy 10 to 50 mg/kg i p)
desmethylimipramine (Pertofran® Geigy 2 to 25 mg/kg i p)
trimeprimine maleate (Surmontil® Leo 50 mg/kg i p)
Ro4 6011 (5 /3 methylaminopropyliden/ dibenzo /a e/ cyclohepta /1 3 5/ trien Hoffman La Roche 10 to 50 mg/kg i p)
amitriptyline (Saroten® Lundbeck 10 to 50 mg/kg i p)
nortriptyline (Lundbeck 10 to 50 mg/kg i p)
protriptyline (5 /3 methylaminopropyl/ 5H dibenzo /a d/ cycloheptene Merck Sharp & Dohme 0 5 to 25 mg/kg i p)
promazine (Protactyl® Ferrosan 10 to 50 mg/kg i p)
chlorpromazine (Hibernal® Leo 10 to 50 mg/kg i p)
chlorprothixene (Truxal®, Lundbeck 10 to 50 mg/kg i p)
levomepromazine (Nozinan® Leo 50 mg/kg i p)
prometazine (Lergigan® Recip 50 mg/kg i p)
chlorpheniramine maleate (Allergisan® Pharmacia 10 mg/kg i p)
tripelenamine (Pyribenzamin® Ciba 10 mg/kg i p)

Tetrabenazine (Hoffmann La Roche 50 mg/kg i p) was dissolved in 0 9 per cent sodium chloride with the help of a minimal amount of hydro

chloric acid and gentle warming The solution was carefully brought to pH 4 to 5 by the addition of potassium bicarbonate powder and injected immediately

Haloperidol (Haldol[®] Leo 25 mg/kg i p) was dissolved by adding equivalent amounts of concentrated lactic acid and boiling water After 24 h in the dark the solution was brought to pH 4 with solid sodium acetate

H 22/54 (α propyl 3 4 dihydroxyphenylacetamide 500 mg/kg i p) and H 33/07 (α ethoxy 2 3 dihydroxyphenylacetamide 500 mg/kg i p) were dissolved in 0 9 per cent sodium chloride

Sympathetic denervation and decentralization Denervation of the rat iris was performed by bilateral extirpation of the superior cervical ganglia together with 5 to 6 mm of the sympathetic trunk and 1 to 2 mm of the postganglionic nerves under ether or pentobarbital anaesthesia Decentralization was performed unilaterally by cutting the preganglionic trunk 3 to 4 mm proximal to the upper cervical ganglion likewise under ether or pentobarbital anaesthesia In both cases ptosis on the operated sides after awakening from the anaesthesia was taken as a sign that the operation had succeeded

Sympathetic stimulation The sympathetic nerves to one of the irises were stimulated in rats by electric stimulation of the preganglionic trunk The vagal nerve together with the sympathetic trunk was carefully dissected free from the carotid artery under a Zeiss operation microscope (magnification ×10) The vagal nerve was cut 1 to 2 mm above the carotid bifurcation and both nerves were cut at the level of the clavicle after a thin thread had been tied around the nerves above the site of lesion The stimulation was mediated by a pair of platinum electrodes 3 to 4 mm apart which were isolated by a thin layer of varnish (Voltalac 435 Standard Varnish AB Gothenburg Sweden) except on the inner surface of the bend The nerves were protected against drying by paraffin oil The Kistner Lab Stimulator 400 used was slightly modified to give biphasic and approximately rectangular impulses The duration of the impulses was about 10 msec and the frequency 10 to 20/sec The voltage was chosen to give a supramaximal stimulation as judged by the dilatation of the pupil and protrusion of the eye ball in normal animals (3 to 6 V) Stimulation was performed under pentobarbital anaesthesia (30 to 35 mg/kg i p) for 10 to 80 min

Other procedures To avoid decarboxylation in the liver intestines and kidneys (Holtz Heise and Lüdtke 1938 Holtz Credner and Rein

HOLD 1939 HOLTZ PEINHOLD and CREDNER 1939) the amino acids were given on occasion intravenously to animals in which the renal and intestinal arteries—or the abdominal aorta just below the diaphragm—had been ligated under pentobarbital anaesthesia (30 to 35 mg/kg i p)

PRINCIPLES FOR THE EVALUATION OF RESULTS One and the same treatment was always performed on at least two separate occasions and generally to two animals at the same time When the effects of different treatment were to be compared the irises from the different animals were always treated in the same way and in the same reaction vessel The irises were sometimes examined without knowing the treatment given to the animal

The specific fluorescence was subjectively evaluated in comparison with that of the control specimens into the four primary grades ' weak moderate strong and very strong These grades are represented in the tables by 1 to 4 plus symbols respectively (see key to the tables page 6) Since certain variations were observed between different animals treated in the same way it proved necessary to introduce certain intermediate grades by putting the last plus in parantheses The fluorescence of the terminals in a normal animal after an optimal reaction is characterized as strong (+ + +)

RESULTS AND DISCUSSIONS

I COMMENTS ON THE TECHNIQUE USED IN PREPARING THE IRIS AND THE FORMALDEHYDE TREATMENT

The present experiments required a high degree of reproducibility and it proved necessary to work out highly standardized and well defined experimental conditions

The following three points appear to be of primary importance the dissection and stretching of the iris the drying of the iris and the formaldehyde treatment

Dissection stretching and initial drying of the iris

The influence of time on the post mortem changes was investigated Rat irises were dissected as rapidly as possible after killing and then immediately frozen in accordance with the freeze drying technique or prepared as whole mounts and dried at room temperature over phosphorus pentoxide In normal animals the adrenergic nerves in the whole mounts showed the same morphology and the same distinct fluorescence with no signs of transmitter diffusion as in the freeze dried specimens (see chapter III) In other experiments the eyes were left *in situ* for various times of up to one hour after killing or immediately removed but kept at +37 C in a moist atmosphere for the same times Whole mounts of the irises were then prepared No obvious fluorescence decrease or other postmortal changes were observed in the nerves of normal animals According to FALCK (1962) a consistent and satisfactory demonstration of the adrenergic terminals is best obtained if the rat irises are excised 10 to 15 min after sacrifice This could not be confirmed in the present work For post mortem changes in animals injected with CA see chapter V

When the iris has been removed the initial drying which serves to attach the tissue to the slide must be performed as quickly as possible (within 1 min) to avoid any diffusion of the NA or other undesirable change in the adrenergic nerves at this stage The preliminary drying is facilitated by drawing the iris to a dry place on the slide and stretching it The latter

step is important for obtaining optimum drying conditions and a minimum of background fluorescence Also the density of the adrenergic plexus will depend on how far the iris is extended Stretching must be maximal during the initial drying but within the physiological limits of the relaxed muscles otherwise the iris will rupture A well stretched rat iris covers a surface of about 3 × 15 mm Its background fluorescence is minimal over the dilator zone and only a little bit stronger in the sphincter zone It seemed to be of no importance which side of the iris was turned upwards

Mouse iris—due to its thinness—dries faster than rat iris and requires more careful and rapid handling

Iris can be very well prepared by a skilful technical assistant after about two weeks training

Drying procedure

The water content of a tissue at the sites of the histochemical reaction has proved to be extremely critical (HAMBERGER MALMFORS and SACHS 1965) It is therefore of great importance to standardize the drying of the whole iris mounts in such a way that the tissue will have a low and fairly constant water content A simple drying of the iris in the room air will give inconstant results owing to the varying humidity of the air If the relative humidity is higher than about 50 per cent too much water is retained which may cause the tissue CA or the fluorescent products formed on formaldehyde treatment to diffuse It was found that drying over phosphorus pentoxide for at least 1 h is necessary to obtain satisfactory results Further drying for up to 24 h seemed to have no significant effect on the histochemical reaction and the whole iris mounts can consequently be stored in the desiccator over phosphorus pentoxide until the formaldehyde treatment is performed (usually within 6 h but sometimes up to 24 h after the preparation) If they are stored in the room air NA will diffuse out from the nerves especially at high relative humidities (more than 50 per cent)

The iris technique used has been found to give inconstant results when the laboratory air has a relative humidity (at 20 to 24 C) of over 70 per cent This seems to be partly due to a rapid adsorption of excessive water by the tissue under these conditions

Formaldehyde treatment

During the formaldehyde treatment—and this is the crucial step in the histochemical method—the CA first condense with formaldehyde to

6 7 dihydroxy 1 2 3 4 tetrahydroisoquinolines which are then in a protein catalyzed reaction dehydrogenated to intensely fluorescent 3 4 dihydro isoquinolines (CORRODI and HILLARP 1963 1964) These reactions require the presence of some water If too much water is present however the amines or their fluorescent products can diffuse It has been found both in the present study and in studies by other investigators in this laboratory that to obtain optimal results it is of great importance for the water content at the site of reaction in the tissue to be nearly as high as can be tolerated without causing diffusion

Under the conditions used in the present work the most important source of water is the paraformaldehyde The polymer must therefore be standardized as to its content of adsorbed water which is of greater importance than the pyrolysis water (see HAMBERGER MALMFORS and SACHS 1965)

Tests have been made on the irises of normal animals using paraformaldehyde with different contents of adsorbed water This was achieved by equilibration in different atmospheres of constant relative humidity (10 to 90 per cent see HAMBERGER MALMFORS and SACHS 1965) When the formaldehyde gas evolved from the polymer contained but little water (equilibration at 10 to 30 per cent relative humidity) the specific fluorescence was distinct but rather weak The varicosities were relatively very pronounced thanks to the very weakly fluorescent segments between (Fig 11) With increasing amounts of water the fluorescence became stronger (cf above) Optimum humidity was that at which the specific fluorescence was as strong as possible without signs of diffusion from the nerves or varicosities The latter were then pronounced and distinct with a high fluorescence intensity Optimum humidity was generally achieved from paraformaldehyde equilibrated at 70 to 80 per cent relative humidity at 20 to 24 C room temperature for at least one week Good results were obtained even at somewhat lower humidity (equilibration at 50 to 60 per cent relative humidity) When the formaldehyde gas evolved from the polymer contained too much water (equilibration at 90 per cent relative humidity or more) the fluorescence showed a broad indistinct appearance due to the diffusion of NA or the fluorescent product within and/or outside the adrenergic nerves (Fig 12) Diffusion was sometimes limited to the Schwann s sheath (Fig 13) At a very high humidity the specific fluorescence of the nerves completely disappeared while the background fluorescence was slightly stronger It proved to be of value to test out the paraformaldehyde evolving a gas of optimum humidity in this way before the experimental irises were treated This gave constant and optimum results When

a normal rat iris was later treated together with the experimental irises in the same vessel to check the reaction, its fluorescence intensity was about the same as in the test reaction with the same sort of paraformaldehyde

Handling of the formaldehyde treated iris preparations

The unmounted iris preparations could be stored in a dark dry place without any evident change in specific fluorescence for at least one week and with a rather small change (some fading and diffusion together with a slight increase in background fluorescence) up to one month after the reaction In a moist and sunny place the specific fluorescence was destroyed within 24 h Mounting the preparations in Entellan with or without a cover glass produced no immediate change in fluorescence but destruction began within 2 to 3 days

DISCUSSION

The specificity and chemistry of the fluorescence method have been extensively discussed by several authors (FALCK 1962 CORRODI and HILLARP 1963 1964 CORRODI HILLARP and JONSSON 1964 DAHLSTROM and FUXE 1964 *a* NORBERG and HAMBERGER 1964) As the histochemical and pharmacological criteria are fulfilled also for the irises dried over phosphorus pentoxide there is no doubt that the specific fluorescence in the normal iris mounts is derived from the endogenous NA in the adrenergic nerves There was no observable difference between the specific fluorescence in these irises as compared with that in freeze dried specimens It must be remembered however that the air drying procedure involves more severe treatment of the tissue than freeze drying

The sensitivity of the present technique seems sometimes to be somewhat higher than in freeze dried tissues (see further chapter IV and the General Discussion) The chief advantage of the present technique is that the *iris is easily and rapidly prepared and dried The reproducibility of the* method is satisfactory provided that the formaldehyde reaction is checked with a normal iris dried together with the experimental ones

The irises of pigmented animals cannot be used

II. SIGNIFICANCE OF THE MANNER OF ADMINISTRATION OF THE DRUGS (ESPECIALLY THE AMINES) AND THE EFFECTS OF DIFFERENT ANAESTHETICS

The uptake of an amine such as NA or α-methyl NA by an adrenergic nerve fibre must be assumed to depend *inter alia* on the concentration of the amine in the extracellular space surrounding the fibre. This concentration depends on a variety of factors. In the case of an exogenous amine the route and mode of administration, the metabolism, the excretion, the ability to penetrate through biological structures etc. all influence this local concentration and thus also the uptake.

In this chapter there will be studied the influence of the route and mode of administration of NA and α-methyl NA on their uptake by the adrenergic iris nerves. The influence of the anaesthetics—used for technical reasons in most of the experiments—on the uptake mechanism is also investigated.

Systemic administration of NA (or α-methyl NA)

It was found that intravenous injections gave the most constant result but that subcutaneous and intraperitoneal injections could give roughly similar results with doses about ten times as large (see chapter V). No great difference in the uptake-accumulation of NA preceded by nialamide (or of α-methyl NA) was observed in reserpinized animals whether a given dose was administered during 6 sec, 2, 10 or 50 min (Table 1). It seems therefore probable that the uptake-accumulation mechanism of the adrenergic nerves in reserpinized animals—and probably also in normal animals—is largely unaffected even by wide variations in the range of the NA dosage and in the rate of injection. It is also obvious that the uptake-accumulation mechanism is very efficient within a wide range of concentrations (see further the General Discussion).

Unless otherwise stated, the intravenous injections of NA (0.02 to 0.1 mg/kg) were performed during a routine time of 2 min. With this mode of administration the rats tolerated 0.1 mg/kg NA (0.2 mg/kg α-methyl NA) in all cases, 0.2 mg/kg NA (0.5 mg/kg α-methyl NA) in most cases and 0.5 mg/kg NA (1 mg/kg α-methyl NA) in some cases. The corresponding doses for intraperitoneal injections were about ten times higher.

TABLE 1 *Effect on the fluorescence intensity of the adrenergic nerves from different rates of administration of α methyl NA or NA preceded by nialamide[1] to reserpinized animals*

(Half of the animals were treated with α methyl NA)

	Total dose (mg/kg)			
Infusion time (min)	Infusion rate (mg/kg min)	0 02	Infusion rate (mg/kg min)	0 1
0 1	200	+(+) (6)	1000	+ + + (6)
2	10	+(+) (4)	50	+ + + (4)
10	2	+ (4)	10	+ +(+) (4)
50	0 4	+ (6)	2	+ +(+) (6)

Intraocular injections

No great reflux of the injected fluid was observed when the puncture was made through the sclera As the volume of the aqueous humor in normal rat has been estimated—geometrically and by maximal acute suction with a microlitre syringe—at about 20 µl and as the volume exchanged was 10 µl the injected fluid was diluted about twice and the initial concentration in the anterior chamber consequently became half that of the injected fluid To exclude interference from the disturbance in intraocular pressure the dilution of the aqueous humor and the solvent one of the eyes was usually given an identical amount of the solvent alone to serve as a control None of these factors have so far been found to have any marked influence on results

The rate of disappearance of the injected drugs from the anterior chamber is as yet unknown There is some evidence (see chapter V) that the NA is reduced to about one tenth within 15 min (Table 10) These observations however must be viewed with some caution as they do not offer any objectively quantifiable data

Effects of different anaesthetics

No difference was found in the uptake accumulation of NA preceded by nialamide (or of α methyl NA) in reserpinized animals (5 mg/kg 18 h before the (A) as between non anaesthetized animals and animals anaesthetized with ether pentobarbital or urethane for the intraperitoneal injection of NA Nor was there any obvious difference in uptake accumulation in animals anaesthetized with ether pentobarbital or urethane for

slow injections (2 min) or infusions (10 min) of different doses of NA preceded by nialamide (or of α methyl NA)

There is thus strong evidence that the anaesthetics used have no or very little influence on the uptake accumulation mechanism in reserpinized animals—and probably also in normal animals—as studied by the present technique This is in complete accordance with other investigations in the same field but using other methods (MUSCHOLL 1960 WHITBY AXELROD and WEIL MALHERBE 1961 CROUT 1964)

DISCUSSION

There seems to be no doubt that the CA are taken up and accumulated in the same way at the doses used as when tracer doses are given as the uptake concentration seems to be independent of dosage within the range used The same result was achieved also with other techniques (IVERSEN 1963 CROUT 1964)

It is clear that the concentration of released NA just outside the adrenergic nerves is far above that in the blood (HAGGENDAL 1963 HILLARP personal communication) It can thus be assumed that the lowest local concentrations used in the anterior chamber of the eye fall within that range Another advantage of intraocular application is the possibility of directly comparing the local administrations with *in vitro* experiments

III THE NORMAL IRIS AND ITS ADRENERGIC NERVES

The sympathetic adrenergic innervation of the iris has been described in detail in previous papers (MALMFORS 1965 MALMFORS and SACHS 1965a and b) In the first of these studies it was concluded that also the sphincter has an adrenergic innervation

Direct studies of adrenergic nerves with the use of the iris technique cannot however be made nor the results understood without a basic knowledge of the morphology and construction of the adrenergic innervation apparatus in the iris The most important features of this apparatus are therefore described below on the basis of findings obtained under normal and optimal conditions for the fluorescence method The adrenergic nerves of the iris have been studied most extensively in rat The innervation in mouse and guinea pig will also be described although mainly with respect to deviations from the rat

Rat iris

In the normal rat iris treated with formaldehyde gas of optimal humidity the adrenergic nerves display a green to yellow green specific fluorescence of varying intensity derived from 4 6 7 trihydroxy 3 4 dihydroisoquinoline the reaction product formed from the endogenous intraneuronal NA (CORRODI and HILLARP 1963 1964) There were no obvious differences between the appearance of the nerves in irises dried over phosphorous pentoxide and that in freeze dried specimens The simple drying procedure at room temperature thus does not produce any serious changes in the morphology of the adrenergic nerves as revealed by the fluorescence method (see chapter I) This agrees with other observations (FALCK 1962 NORBERG and HAMBERGER 1964)

The innervation apparatus of the dilator muscle appears as a dense finely meshed two-dimensional network each strand of which usually contains two or more fine varicose terminals running together (Fig 8 10 22 34) The terminals in this ground plexus show a strong and distinct fluorescence over the whole iris but are often best studied in the peripheral part near the ciliary body where the weak diffuse background fluorescence (a dirty yellow green) is often almost entirely absent Careful examination of the cross-sections from freeze-dried irises has shown that the plexus is directly

superimposed on the corneal surface of the muscle cells which form a single layer and that the terminals do not penetrate in between these cells (MALMFORS 1965) This has been confirmed by electron microscopy (NILSSON 1964)

A typical and fairly dense adrenergic ground plexus is present also in the walls of the small arterioles (Fig 6) which run in the iris stroma on the corneal side of the dilator muscle

The adrenergic plexus of the sphincter has a somewhat different appear ance and there is a distinct boundary between the sphincter and dilator plexus (Fig 7) In the sphincter the terminals run for the most part singly in the same direction as the muscle cells and are present not only on the surface but can be seen in sections of freeze dried irises to penetrate in between the cells It therefore seems probable that these terminals really innervate the muscle cells of the spincter (see MALMFORS 1965)

With the use of the iris technique it has become possible for the first time to obtain direct and detailed information as to the construction of this complex adrenergic apparatus particulary in the rat iris by studying the adrenergic nerves remaining in the irises after an almost complete sympathetic denervation (MALMFORS and SACHS 1965b) The spread and different parts of the axons of adrenergic neurons in the rat iris are schema tically illustrated in Fig 1 and described briefly in the accompanying text

The main axons (Fig 4) which are most easily found in nerve trunks near the ciliary body appear to be uniformly thick (0 75 to 1 μ in diameter) and show in normal animals a weak uniform fluorescence They give off branches of roughly identical appearance—the preterminal axons—which form small nerve bundles These bundles are not easily observed owing to the thinness (about 0 5 μ) and very weak fluorescence of the axons here (Fig 18) The non terminal axons—both the main and the pre terminal axons—can be readily observed however if only their amine content is raised (see chapter V Fig 21 23) The preterminal axons are finally and suddenly transformed into terminals (see Fig 1) which form the ground plexus the real innervation structure (cf NORBERG and HAMBERGER 1964) The terminals have a highly characteristic appearance with abundant small somewhat elongated enlargements or varicosities which exhibit a strong fluorescence These varicosities are on the average 1 μ thick and spaced somewhat irregularly along the terminal (about 20 per 100 μ fibre length) The segments of fibre between these structures are thin (about 0 2 to 0 5 μ) and display a fairly weak fluorescence

Most of the terminals end with an enlargement of the same appearance as the varicosities Some however end without any marked swelling Even

in the intact iris terminals may be seen which appear to end with an enlargement but it is difficult to decide whether this is a true anatomical ending or has been artificially produced during the stretching of the tissues

Following unilateral cervical sympathectomy all the adrenergic nerves in the iris on the same side 'disappear' within 36 h (for details see chapter VII) Section of the preganglionic trunk on the other hand gives no obvious changes in the fluorescence This confirms the classical view that the innervation is of unilateral sympathetic origin and that most—if not all—of the ganglion cells supplying the iris are located in the superior cervical ganglion It must be noted however that in a few per cent of rats one or two of these cells are located more peripherally—in all probability in the postganglionic nerves—and remain intact after removal of this ganglion (Malmfors and Sachs 1965*a* and *b*)

Mouse iris

The adrenergic innervation apparatus in mouse differs from that in rat on two interesting points Almost everywhere in the plexus on the dilator muscle the strands of the network contain only a single terminal (Fig 30) The varicosities of the terminals are very prominent (on the average about 1 5 μ thick)

The mouse iris is thus eminently suitable for studies on the adrenergic terminal It is also thinner than the rat iris and shows less background fluorescence

Guinea pig iris

The adrenergic innervation apparatus is very similar to that in rat (Fig 9) It is more difficult however to examine the nerves in the guinea pig iris due e g to a higher background fluorescence

DISCUSSION

Studies on adrenergic nerves with the use of the fluorescence method in this laboratory have furnished strong evidence for the view (Hillarp 1946 1959) that the fine terminal axon ramifications are the true terminals that store and release the transmitter (Norberg and Hamberger 1964 Malmfors and Sachs 1965*a* and *b*) With the use of the iris technique it has in [illegible] t been [illegible] to obtain strong direct evidence that the ab[illegible] [illegible] along the entire length of the terminal are th[illegible] [illegible] [illegible] for the synthesis storage and release of [illegible] ter ([illegible] VIII and Malmfors [illegible])

It has been shown that the specific fluorescence observed in the peripheral adrenergic neuron is due to NA and that other CA and DOPA exist in amounts of no significance from the histochemical point of view (NORBERG and HAMBERGER 1964) From the fluorescence intensity it has been concluded (NORBERG and HAMBERGER 1964) that the ganglion cell bodies and postganglionic fibres in the peripheral adrenergic nerves have a low NA content (about 10 to 100 μg/g wet wt) while the preterminal axons have somewhat higher concentrations (probably up to 500 μg/g) In the terminals which to judge from the distribution of the fluorescence contain practically all the transmitter stored in these neurons the varicosities have extremely high concentrations (probably in the order of 10 000 μg/g) and are very abundant They are consequently the real site of transmitter storage as the thin segments between have much lower concentrations and are sometimes only barely visible

There is strong evidence that the transmitter is bound—at least for the most part—to specific storage granules and does not normally exist free in the cytoplasm (see the General Discussion) These granules are formed in the cell bodies and then transported down to the terminals (DAHLSTRÖM and FUXE 1964b and c DAHLSTRÖM 1965 DAHLSTRÖM FUXE and HILLARP 1965)

As concluded by NORBERG and HAMBERGER (1964) the intraneuronal distribution of the NA in normal animals may well reflect the distribution of the transmitter granules (see the General Discussion) It is therefore probable that the non terminal part of the postganglionic axon contains only a few amine granules while extremely high numbers of granules are accumulated within the varicosities of the terminals This is supported by electron microscopical observations on the intraneuronal distribution of vesicles (DEROBERTIS 1962 ELFVIN 1963) In this connection it must be emphasised that not only the granule bound NA—as suggested by CSILLIK (1964)—but also the CA existing extragranularly in the cytoplasm of the adrenergic nerves can be readily demonstrated by the fluorescence method used (see NORBERG and HAMBERGER 1964 chapters V and VII and the General Discussion)

IV DEPLETION AND RECOVERY OF THE ENDOGENOUS NA AFTER SYSTEMIC OR LOCAL RESERPINE TREATMENT AND THE EFFECTS OF MAOI AND BRETYLIUM ON DEPLETION

The behaviour of the endogenous NA in the adrenergic nerves of different organs following systemic treatment with reserpine has been studied with both biochemical and histochemical methods on several different occasions It has been well established that reserpine causes a disappearance of the NA which then begins to reappear within about 2 to 4 days after the reserpine administration (CARLSSON ROSENGREN BERTLER and NILSSON 1957 ANDÉN MAGNUSSON and WALDECK 1964 BHAGAT and SHIDEMAN 1964 HAGGENDAL and LINDQVIST 1964*a* and *b*) In the rat, 0 5 mg/kg reserpine has been found necessary for an almost complete depletion of NA (FALCK 1962 NORBERG 1965*a*)

There is very strong evidence that this depletion is due to inhibition of the uptake mechanism of the amine granules in the adrenergic nerves so that the latter are depleted of their NA (see the General Discussion)

With the present technique it is possible to study the depletion and recovery following reserpine treatment in more detail since the disappearance and reappearance of NA can be observed in every separate nerve fibre and actually in each individual part of the nerve even at very low levels of NA Also the intraocular administration makes it possible to study the local effects of reserpine in concentrations that are much more varied than with systemic administration This chapter will report the main features of depletion and recovery of NA following reserpine treatment as revealed by the present technique

Depletion of endogenous NA by the systemic administration of different doses of reserpine

(See *Table 2 and Fig 43*)

When reserpine was administered to the animals (rats and mice) 0 5 to 24 h before death the specific fluorescence of the adrenergic nerves in the whole mounts of iris was reduced or had completely disappeared The degree of reduction was dependent both on the dose and on the interval between administration and death The fluorescence had thus in nearly all

cases disappeared 4 h after 10 mg/kg i p while fluorescence was still left 8 h (but not 16 h) after 1 mg/kg The *first* signs of a decrease in fluorescence intensity were found 30 min after an intravenous injection of 10 mg/kg or 1 h after an equal dose given intraperitoneally There followed a gradual decrease—somewhat more rapid after the intravenous injection—until all fluorescence had disappeared After 24 h 0 25 mg/kg i p had caused a slight and 0 5 mg/kg a strong reduction in fluorescence while 1 mg/kg nearly always caused a complete disappearance 2 5 mg/kg or more always caused a complete disappearance of the fluorescence

As fluorescence declined the varicosities became relatively more pronounced as the segments between became very weak (Fig 14 15 42b) As depletion came to an end the fluorescent fibres disappeared somewhat unequally over the iris leaving to the last individual systems of branching terminals of moderate fluorescence (Fig 16) This phenomenon was most pronounced at low doses (1 mg/kg) The reason for the delayed depletion of certain neurons is obscure but differences in e g impulse activity or MAO activity are conceivable (see further the General Discussion) as are variations in transmitter content

The fluorescence of the non terminal axons disappeared somewhat earlier than that of the terminals

Recovery of endogenous NA after the systemic administration of different doses of reserpine

(*See Table 2 and Fig 43*)

When the animals (rats and mice) were killed 32 to 96 h after the administration of reserpine in sufficient doses to cause a complete depletion the fluorescence had begun to reappear Already at 32 h after 1 mg/kg or 40 h after 10 mg/kg there was a weak but evident reappearance of the specific fluorescence which was of roughly the same appearance and distribution as the unequally distributed fluorescence towards the end of depletion 40 h after the low dose and 48 h after the large dose most of the fibres had regained a weak fluorescence which was mainly located to the varicosities These had thus become relatively more pronounced in the same way as during depletion (Fig 17) 72 h after 1 mg/kg and 96 h or more after 10 mg kg the fluorescence had returned to about the same intensity as that of a normal iris

The fluorescence of the non terminal axons reappeared at about the same time as that of the terminals

No significant difference in the depletion and recovery of the fluorescence was observed as between the commercial solution of reserpine and a freshly made solution of pure reserpine

Depletion and recovery were of about the same magnitude in mouse iris as in rat

Depletion and recovery of endogenous NA in the iris after the intraocular administration of reserpine

(See Table 2 and Fig 43)

As late as 2 h after an intraocular administration of 2 μg reserpine phosphate in the anterior chamber there was still left a weak fluorescence which had the same appearance as that of the reduced fluorescence following the systemic administration of reserpine By 4 h after the local administration this fluorescence had completely disappeared The background fluorescence had increased presumably due to reserpine itself or some of its metabolites which can become fluorescent after formaldehyde treatment (HILLARP personal communication) At 24 h after local intraocular administration 0 1 μg produced a fairly strong reduction and 0 5 μg an almost complete reduction of the fluorescence of the terminals

The first signs of the terminals reappearing after the intraocular administration of 10 μg reserpine were observed 96 h later and then only in the peripheral parts of the iris 6 days after treatment however there was a weak reappearance over the entire iris The fluorescence was not of normal intensity however even after a further 6 days

The background fluorescence was not markedly increased 24 h or more after the administration not even following the maximum dose

The non terminal axons disappeared at about the same time as the terminals but regained an almost normal fluorescence intensity much sooner Weak non terminal axons with a large diameter (main axons) could thus be found more or less alone in the whole mounts of iris 24 h after 2 μg or 48 h after 10 μg and by the time the terminals were reappearing the non terminal axons had become relatively pronounced

In the control eye the solvent (5 5 per cent glucose) produced no change whatsoever in fluorescence

As a control certain other animals given reserpine (10 μg) intraocularly at different times (24 to 96 h) before death were treated with NA preceded by nialamide or with α methyl NA to see if the uptake accumulation mechanism of the axon membrane was intact Since a restitution of the specific fluorescence was observed as after systemic pretreatment with reserpine

Table 2 *Depletion and recovery of the endogenous NA after reserpine treatment*

Time (h)	Dose (mg/kg) i.p. 0.1	0.2	0.5	1	2.5	5	10	i.v. 10	Dose (µg/eye) 0.1	0	2	10
0.5							+ + + (10)	+ + (2)				
1				+ + + ( )			+ + (11)	+ (4)				
2				+ + ( )			+ (13)	+ (2)			+ ( )	
3				+ + (2)			(+) (8)	(+) ( )				
4				+ (8) ( )*			– ( ) ( )*	– (3)			– (1)	
9				(+) (6) ( )*			– (8) ( )*					
16			(+) (4)	– (8) (?)*	( )		– (4)					
24	+ + + (6)	+ + (4)	+ (10)	(+) (9) (2)*	– (9)	– (4)	– (16)		+ ( )	(+) (3)	– (3)	– ( )
32			+ ( )	+ (10) (4)*	+ (3)	(+) (4)	– (17) ( )*	– ( )				
40			+ + (?)	+ + (11) (5)*		+ (1)	+ (14) (6)*	+ (2)				
48			+ + (?)	+ + (6) ( )*		+ + (1)	+ (15) (2)*	+ + (?)				– (3)
56			+ + + (?)	+ + (6) (2)*			+ + (6) (?)*	+ + (2)				
[illegible]			+ + + ( )	+ + + (4) ( )			+ + (8) (?)*					
96				+ + + (2)			+ + + (6)					(+) (3)
144							+ + + (6)					(+) (1)
288												+ + (3)

(see chapter V), it was concluded that the delayed reappearance was at least not mainly due to disturbances in the uptake accumulation mechanism of the axon membrane

Inhibition of the reserpine effect and retardation of the depletion of NA by MAOI (nialamide) and bretylium

(See Table 3)

When nialamide (100 mg/kg i p) was administered simultaneously with reserpine (10 mg/kg i p) the reserpine induced reduction of the fluorescence was clearly diminished 6 h after the injection When the nialamide was given 1 or 3 h before reserpine the fluorescence intensity 6 h after the reserpine administration was the same or even higher than in an iris from an untreated animal 24 h after the reserpine treatment the fluorescence had almost entirely disappeared in the animals treated simultaneously with nialamide while a moderate fluorescence intensity was still observed in the animals treated with nialamide 3 h before The appearance of the fluorescence remaining after nialamide and reserpine was essentially the same as that after reserpine alone at corresponding intensity Sometimes however the fluorescence of the non terminal axons appeared to be slightly increased

Certain other MAOI (pheniprazine and pargyline) showed the same effect as nialamide but these experiments are not reported in detail in this paper as very few animals survived

Bretylium (50 mg/kg) given simultaneously with or 1 h before reserpine had the same sort of influence as nialamide on the reduction of the fluorescence but to a slightly lesser extent

TABLE 3 *Effect of nialamide and bretylium on the reserpine induced depletion of endogenous NA*

		Reserpine (10 mg/kg i p) Time before death (h)		
Drug before reserpine	Time before reserpine (h)	3	6	24
Nialamide (100 mg/kg)	0	+ +(+) (5)	+(+) (4)	(+) (4)
	1		+ + + (4)	+ (5)
	3		+ + + (5)	+ + (4)
Bretylium (50 mg/kg)	0	+ + (2)	+(+) (3)	
	1	+ + + (2)	+ + (3)	
–	–	(+) (8)	– (4)	–(10)

DISCUSSION

The action of reserpine will be discussed more comprehensively in the General Discussion. Only certain particularly important features directly connected with the results reported in this chapter will be considered here.

The results here obtained by the iris technique agree very well with the results obtained by other techniques. It is true that the fluorescence method gives no quantitative data. But since e.g. the onset of recovery is discovered at about the same time with the histochemical method as with biochemical methods, the weakest specific fluorescence observed must represent very small concentrations of NA (see further the General Discussion). The slight deviation between the results of the present study and those obtained by NORBERG (1965 *a*) with the fluorescence method applied to freeze dried specimens (e.g. the submaxillary gland and the vas deferens) may be due to the use of different tissues but could also suggest a somewhat higher sensitivity with the present technique.

The depletion of NA took place relatively slowly, even after a high dose of reserpine (systemically or locally). This gives no support to the assumption of a direct releasing action on the transmitter as proposed e.g. by CSILLIK (1964), whose results could not be reproduced in our laboratory (HAMBERGER, personal communication).

The observation of distinct individual systems of branching terminals during the final stage of reserpine induced depletion and the initial stage of recovery suggests markedly higher inter- than intraneuronal variations. This could be related to differences in metabolic and/or functional activity. No obvious differences were observed between the terminals innervating the dilator, the sphincter and the vessels.

It is probable that the NA content during depletion is reduced at about the same rate in the varicosities as in the segments between, since the varicosities retained their relatively pronounced fluorescence even with very low amounts of transmitter remaining (cf. the depletion following stimulation, chapter VIII). The non terminal axons seem to be depleted at about the same rate as the segments between the varicosities. Since it is assumed that the transmitter is localized for the most part to the amine granules—both in the terminals and in the non terminal axons—(see chapter III and the General Discussion) and that reserpine acts primarily on these granules (CARLSSON 1965 *a*, DAHLSTRÖM, FUXE and HILLARP 1965), it would appear that the granules are depleted at about the same rate regardless of their intraaxonal localization.

There is reason to believe that the transmitter reappearing after deple-

tion by reserpine is also localized mainly to amine granules since it had e g the same intraneuronal distribution as normally The question arises whether these are the previously depleted granules or whether the latter have been exchanged for newly formed amine granules transported down from the cell body It seems improbable that the recovery following systemic treatment with reserpine could be due solely to a refilling by new granules since preliminary data have shown that only about 3 per cent of the amine granules in the terminals are replaced every day (DAHLSTRÖM and HÄGGENDAL 1965*a*) It is probable rather that the blockade of the amine granules by systemically administered reserpine is partly reversible This is further supported by the significant difference between the rates of NA reappearance after different doses and the uniform reappearance of the transmitter in different parts of one and the same system of branching terminals over the entire iris (including the non terminal axons) On the other hand the recovery after local administration which is much slower may well coincide with a refilling of newly formed amine granules The irregular restitution of the transmitter in the terminals over the whole iris and the relatively rapid restitution of the non terminal axons likewise argue for an irreversible blockade of the granules in this case

The influence of MAOI and bretylium on the effect of reserpine is known from biochemical experiments (CARLSSON ROSENGREN BERTLER and NILSSON 1957 ARNOLD MCAULIFF SOBELL and ARCHER 1963 BENMILOUD and EULER 1963 POTTER and AXELROD 1963*a* KOPIN 1964) The results of the present study are in accordance with these investigations There was no redistribution of fluorescence during the delayed depletion as compared to that during the depletion after reserpine alone

V UPTAKE ACCUMULATION STORAGE AND RELEASE OF EXOGENOUS PRIMARY MONOAMINES AND CERTAIN OF THEIR PRECURSORS

As mentioned above it has been demonstrated that NA and DA can be taken up by adrenergic nerves and that this uptake is not inhibited by reserpine It has also been shown that CA taken up are rapidly metabolized by MAO It is therefore necessary satisfactorily to inhibit the MAO with some MAOI e g nialamide in order to be able to study the uptake and accumulation mechanism more comprehensively It is also possible to use the α methylated derivatives of NA and DA which are not readily metabolized by MAO (CARLSSON and LINDQVIST 1962) but give the same fluorescence reaction after treatment with formaldehyde gas as the non methylated compounds (HILLARP personal communication)

Most of the experiments reported in this section were performed on reserpinized animals since the fluorescence intensity in normal animals is too high to permit observation of the uptake and accumulation of small amounts of CA However animals not treated with reserpine were also used in these studies Particularly the non terminal axons which normally have only a weak fluorescence can be successfully studied in such animals

It has been found by chemical methods that 5 HT is taken up by sympathetically innervated tissues (AXELROD and INSCOE 1963)

It has been shown that L-DOPA the direct precursor to DA causes an elevation of the NA content in the brain (CARLSSON LINDQVIST MAGNUSSON and WALDECK 1958) presumably through its being taken up by adrenergic neurons and decarboxylated to DA which then is rapidly converted to NA in the amine granules (KIRSHNER 1957 POTTER and AXELROD 1963 *b*) The systemic injection of L-DOPA produces signs of increased formation of NA or DA also in the peripheral adrenergic neurons (PENNEFATHER and RAND 1960) but an extraneuronal decarboxylation of L DOPA and uptake of DA cannot here be excluded This potential source of error has been reduced in the present study in three ways

a) by administering the L-DOPA intraocularly

b) by preventing decarboxylation of the L DOPA in the kidneys liver and intestines by ligating the arteries of these organs previous to intravenous injection

c) by inhibiting the uptake of extraneuronally formed DA by e g desmethylimipramine (see chapter IX)

The same precautions have been taken with D DOPA

With decarboxylase inhibitors it is possible to distinguish between accumulation of the amino acid—which also gives the fluorescence reaction—and accumulation of DA or NA As these amines are metabolised by MAO pretreatment with a MAOI is necessary however for this purpose It has been shown that the α methylated derivative of DOPA is probably taken up by adrenergic nerves but that it is not decarboxylated to its corresponding amine α methyl DA as easily as L DOPA as it has a certain decarboxylase inhibiting effect (OATES GILLESPIE UDENFRIEND and SJOERDSMA 1960 PUTTER and KRONEBERG)

DOPS and 5 HTP, which are the direct precursors of NA and 5 HT respectively have also been used in combination with MAOI in certain of the studies reported in this chapter

The uptake accumulation storage and release of the above mentioned compounds will be described in this chapter with regard to the effects of different doses and different types of administration and in respect of the duration of the storage of the accumulated amines Results as revealed by the fluorescence method are described in the text and presented schematically in Tables 4 to 17 and in Fig 44 45

Non reserpinized animals

When NA (0 02 to 0 5 mg/kg i v —Table 4 or 10^{-6} to 10^{-4} *M* initial concentration intraocularly—Table 5) was administered 5 min to 2 h before death there was a clear increase in the fluorescence intensity of the adrenergic terminals This was seen as early as 5 min after 0 1 mg/kg or more given systemically 10^{-5} *M* or more given locally After the systemic treatment the non terminal axons showed only a slight increase in fluores

TABLE 4 *The fluorescence intensity of the adrenergic nerves after the administration of NA i v to normal animals*

Dose (mg kg)	Time before death (h)			
	0 1	0 5	1	
0 02	( )			
0 1	((	+ +( ) (4)	+ + + (4)	+ + + (3)
0	(–	+ (3)		

TABLE 5 *The fluorescence intensity of the adrenergic nerves after the administration of NA intraocularly to normal animals*

Initial concentration (M)	Time before death (h) 0 1	0 25	0 5	5 min post mortem Preparation 20 min post mortem
10^{-6}	+ + +(+) ([illegible])			
10^{-5}	+ + + + ([illegible])			+ + +(+) (2)
10^{-4}	+ + + + ([illegible])	+ + + + (2)	+ + +(+) ([illegible])	+ + + + ([illegible])

cence even if the iris was left *in situ* for 15 min post mortem Not even the terminals showed any significant increase in fluorescence intensity when the preparation was made 15 min post mortem which after a sufficiently high dose of NA has been found to produce a weak restitution of the fluorescence in reserpinized animals (see below) After the local administration however there was a further increase in the fluorescence intensity post mortem in both the terminals and the non terminal axons This was true even when these injections were made 5 min post mortem 15 min before preparation

The increase in fluorescence intensity disappeared rapidly particularly in the systemically treated animals where fluorescence was normal again 1 h after the NA administration (see Fig 44)

If the animals were pretreated with nialamide (100 mg/kg) about 2 h before the administration of NA (0 02 to 0 5 mg/kg i v —Table 6—or 10^{-6} to 10^{-4} M initial concentration intraocularly—Table 7) 15 min to 4 h before death (Fig 44) there was a strong increase in fluorescence intensity 15

TABLE 6 *The fluorescence intensity of the adrenergic nerves after the administration of NA i v preceded by nialamide*[1] *to normal animals*

Dose (mg/kg)	Time before death (h) 0 25	1	2	4
0 02	+ + + (4)			
0 1	+ + + + (10)	+ + + + (5)	+ + + + (4)	+ + + + (4)
Compared with nialamide alone (Table 18)	Great increase	Great increase	Slight increase	No increase
0 5	+ + + + (3)[a]			

Died after 10 min

TABLE 7 *The fluorescence intensity of the adrenergic nerves after the administration of NA intraocularly preceded by nialamide[1] to normal animals*

Initial concentration (M)	15 min before death	Compared with nialamide alone (the control eye)
10^{-6}	+ + +(+) (2)	Slight increase
10^{-5}	+ + + + (4)	Great increase
10^{-4}	+ + + + (2)	Great increase

min after 0 1 mg/kg or more systemically or after 10^{-5} M or more locally (This in comparison with nialamide treated controls) The fluorescence intensity was raised in both the terminals and the non terminal axons In the former the segments between the varicosities increased relatively more than the varicosities themselves giving the terminals a somewhat smoother appearance than normal (Fig 23 42c) Even so the varicosities which also increased in intensity were still much higher than the segments between in contrast with the markedly smooth appearance of the terminals after the same treatment in reserpinized animals (see below) In the non terminal axons there was a strong uniform increase in fluorescence along their entire length making them clearly visible and easy to follow The same appearances as described above and roughly the same increases in fluorescence intensity were obtained after treatment with the same doses and concentrations of α methyl NA 15 min before death without any preceding MAOI (Table 8) (This in comparison with untreated controls)

The duration of observable increases in intensity following systemic treatment was difficult to decide particularly in the animals treated with nialamide and NA as nialamide given alone gave a progressively increasing fluorescence (see chapter VII) A comparison of CA treated specimens with control specimens treated in the same reaction vessel showed however

TABLE 8 *The fluorescence intensity of the adrenergic nerves after the administration of α methyl NA i v to normal animals*

Dose (mg/kg)	Time before death (h)				
	0 5	1	2	4	6
0 0	+ + (4)				
0 1	(6)	+ + (3)	+ + + (+) (5)	+ + + (3)	+ + (9)
0	+ (5)				

a noticeable increase in fluorescence 1 h after administration and a slighter increase only after 2 h No increase at all could be observed after 4 h (Fig 44)

Not all animals survived the highest doses particularly those of NA in combination with nialamide Such animals died 5 to 10 min after the beginning of the injection and were then prepared immediately There was also a noticeable increase in fluorescence 15 min after pretreatment with DA (5 mg/kg i v —9 rats) especially when the animals (6) were pretreated with nialamide (100 mg/kg 2 h before the administration of DA) L-DOPA (20 mg/kg i p —8 rats) gave similar results

There was no increase in background fluorescence following NA or α methyl NA and only a very slight increase in animals treated with DA and L DOPA

Corresponding increases in fluorescence intensity were observed also in mice following the administration of NA α methyl NA DA and L-DOPA with or without pretreatment with nialamide (Fig 31) Fluorescence increases *were particularly obvious in the non terminal axons which in the* normal mouse iris have a very weak fluorescence (Fig 30)

The colour of the increased fluorescence was judged subjectively to be the same as in nerves of normal irides

Reserpinized animals

NA (or α methyl NA)

Unless otherwise stated reserpine treatment comprized in this section 5 mg/kg i p given about 15 to 21 h (reported as 18 h) before administration of the substance examined This treatment has been found in all cases to cause a complete disappearance of the specific fluorescence in the iris for at least up to 24 h (see chapter IV)

NA ((–) NA 0 02 to 0 5 mg/kg i v —Table 9—5 mg/kg i p or s c —10 rats—or 10⁻ to 10^{-4} *M* initial concentration intraocularly—Table 10)

TABLE 9 *The intensity of the restituted fluorescence of the adrenergic nerves after the administration of NA i v to reserpinized animals*

	Time before death (h)					
Dose	Preparation at once			Preparation 15 min post mortem		
(mg kg)	0 1	0 25	0 5	0 1	0 25	0 5
0 02	– (9)			– (2)		
0 1	(+) (10) (2)	– (4)	– (4)	+(+) (10) (2)	– (4)	– (4)
0 5	+ (6) (2)	+ (2)		– + + (6) (2)	+ + (2)	

TABLE 10 *The intensity of the restituted fluorescence of the adrenergic nerves after the administration of NA intraocularly to reserpinized animals*[2]

Initial concentration (M)	Time before death (h) 0 1	0 25	1	2	4	5 min post mortem: Preparation 20 min post mortem
			Preparation at once			
10^{-7}	− (2)					− (3)
10^{-6}	− (2)					+ (3)
10^{-5}	(+) (4)	(+) (3)	− (3)			+ + (3)
10^{-4}	+ (6) (2)*	+ (4)	+ (5)	(+) 2	− (2)	+ + + (3)
			Preparation 15 min post mortem			
10^{-7}	+ (2)					
10^{-6}	+ (2)					
10^{-5}	+ + (4)	+ (3)	(+) (3)			
10^{-4}	+ + + (6) (2)*	+ + (4)	+ + (5)	+ (2)	− (2)	

was given to reserpinized animals (not pretreated with MAOI) 5 min to 4 h before death 5 min after the administration of 0 1 mg/kg i v or 10^{-5} *M* intraocularly there was found—if the iris was prepared immediately after death—a very weak specific fluorescence in the adrenergic nerves If however the iris was left *in situ* for about 15 min post mortem the specific fluorescence had increased—after this dose and concentration to a moderate level Even with the highest dose and concentration the fluorescence in the immediately prepared irises was low 5 min after administration In the irises prepared 15 min post mortem however the specific fluorescence intensity had increased to almost the same as in normal iris 15 min after 0 1 mg/kg i v no specific fluorescence could be found either when the irises were prepared and dried immediately after death or when they were prepared 15 min post mortem

With the local administration post mortem restitution of the fluorescence was found after 10^{-6} *M or higher concentrations Post mortem restitution* was observed much longer after local than systemic administration The fluorescence in the immediately prepared irises on the other hand was rather weak but was found up to at least 1 h after the administration of NA

Post mortem restitution seems to be due to the fact that oxidative deamination by MAO cannot take place in the absence of oxygen while at the same time NA is still extraneuronally available for the uptake accumulation mechanism—which is probably localized to the axon membrane—and apparently not as sensitive to anoxia as MAO This agrees with

TABLE 11 *The intensity of the restituted fluorescence of the adrenergic nerves after the administration of NA i.v. preceded by nialamide[1] to reserpinized animals*

Dose (mg/kg)	Time before death (h)						
	0.1	0.5	1	2	4	6	8
0.01		+ (3)					
0.02		+(+) (10) (_)* ( )	+ ( ) ( )* (?)	+ ( ) ( )* (2)	(+) (1)		
0.05		+ + ( )					
0.1	+ + + ( ) ( )* (_)	+ + + (1) (0)* ( )	+ + + (8) (_)* ( )	+ + (3)	+(+) (9) ( )* ( )	+ ( )	(+) ( )
0.2		+ + + (5)					

TABLE 14 *The intensity of the restituted fluorescence of the adrenergic nerves after the administration of α-methyl-NA i.v. to reserpinized animals*[2]

Dose (mg/kg)	Time before death (h)						
	0.1	0.5	1	2	4	6	8
0.01		+ ( )					
0.02		+ (0) (4)* (2)	+ (8) (2)* (_)	(+) (5) (2)* (_)			
0.05		+ + ( )	+(+) (8)	+ (4)			
0.1	+ +(+) ( )	+ + + (13) (10)* (_)	+ +(+) (7) (_)* (3)	+ + (8)	+(+) (11) ( )* (3)	+ (4)	( ) (5)
0.2		+ + + (10)	+ + + (2)		+ + (4)	+ + (?)	

TABLE 12 *The intensity of the restituted fluorescence of the adrenergic nerves after the administration of NA i p or s c preceded by nialamide[1] to reserpinized animals*

Dose (mg/kg)	1 h before death
0 5	+ (3)
1–2	+ + (10)
5	+ + + (5)

other findings that post mortem restitution following systemic administration occurs only during a brief period after the injection The post mortem restitution after local administration on the other hand can serve as an approximate measure of the *in vivo* rate of disappearance of NA injected in the anterior chamber of the eye

Local administrations of NA (10^{-7} to 10^{-4} *M* initial concentration intraocularly) were performed also 5 min post mortem and 15 min before preparation Even in the dead animal there was a fairly good restitution of fluorescence to about the same magnitude as the post mortem restituted fluorescence after the *in vivo* administration of NA (Table 10)

15 min after the intraperitoneal or subcutaneous injections there was a very weak fluorescence in the immediately prepared irises while 15 min post mortem the fluorescence intensity had increased to moderate levels

If MAO was effectively inhibited before the administration of NA ((−) NA 0 01 to 0 2 mg/kg i v —Table 11—0 5 to 5 mg/kg i p or s c—Table 12—or 10^{-9} to 10^{-5} *M* initial concentration intraocularly—Table 13) with e g nialamide (100 mg/kg) administered about 2 h before the NA the restituted fluorescence became stronger than the post mortem restituted fluorescence even immediately after death A rather weak but distinct specific fluorescence was thus found 15 min after only 0 01 mg/kg i v 0 02 mg/kg gave a weak to moderate fluorescence while 0 1 mg/kg caused a restitution of the fluorescence intensity to nearly the same level as in untreated animals As early as 5 min after the latter dose fluorescence had reached about the same intensity as after 15 min No significant increase was observed if the irises were left *in situ* post mortem for 15 min It is thus evident that NA was taken up very rapidly but that the NA available for uptake was reduced within a very short time

1 h after an intraperitoneal or subcutaneous injection the fluorescence was weak after 0 5 mg/kg moderate after 1 to 2 mg/kg and strong after 5 mg/kg

TABLE 13 *The intensity of the restituted fluorescence of the adrenergic nerves after the administration of NA intraocularly preceded by nialamide[1] to reserpinized animals*

Initial concentration (M)	15 min before death	
10^{-9}	(+)	(4)
10^{-8}	+	(9) (3)
10^{-7}	+ +	(10) (3)
10^{-6}	+ + +	(9) (3)
10^{-5}	+ + +(+)	(5) (1)

When NA was administered in the anterior chamber of the eye a distinct but weak fluorescence could be found 15 min after an injection of only about 0 035 ng/eye (initial concentration intraocularly 10^{-8} M) 10^{-7} M gave a moderate and 10^{-6} M (3 5 ng/eye) a strong fluorescence of about the same intensity as in the normal iris (Fig 28)

The time of the spontaneous disappearance of the restituted fluorescence after NA preceded by nialamide was dependent on the dose The weak to moderate fluorescence following 0 02 mg/kg i v remained almost unchanged up to 1 h after the administration and then slowly disappeared during the next 1 to 3 h (Fig 45) while the strong fluorescence following 0 1 mg/kg i v remained almost unchanged up to 2 h after the administration and then slowly disappeared during the next 2 to 6 h (Fig 45)

Following the systemic administration of α methyl NA (0 01 to 0 2 mg/kg i v —Table 14) the restituted fluorescence was of about the same intensity as after the administration of equal doses of NA preceded by nialamide while the restitution after intraocular administration (10^{-8} to 10^{-5} M initial concentration intraocularly—Table 15) was somewhat less The fluorescence obtained after α methyl NA disappeared at about the same time as after NA preceded by nialamide Pretreatment with MAOI (nialamide) did not potentiate the restitution of fluorescence by α methyl NA

No difference was observed in the restituted fluorescence after NA with or without nialamide or α methyl NA after different doses of reserpine administered at different times before the CA The fluorescence was thus the same in animals treated with 1 mg/kg reserpine 24 h before administration of the CA as in animals treated with 5 mg/kg 18 h before or with 10 mg kg 4 h before Nor did a large dose (10 mg/kg i v) of reserpine shortly

TABLE 15 *The intensity of the restituted fluorescence of the adrenergic nerves after the administration of α methyl NA intraocularly to reserpinized animals*

Initial concentration (M)	15 min before death	
10^{-8}	(±)	(2) (4)*
10^{-7}	+	(7) (4)*
10^{-6}	+ +	(6) (3)*
10^{-5}	+ + +	(3)

before (5 min) the administration of the CA appear to influence restitution (see chapter IX)

The distribution and the colour of the restituted fluorescent fibres were the same as with the fluorescent fibres of a normal iris But the appearance of the adrenergic terminals was markedly changed The varicosities looked thinner and the segments between showed a relatively higher fluorescence intensity than in normal terminals The varicosities thus appeared much less prominent giving the entire terminal an almost smooth appearance (Fig 25 33 42*e*) The smooth terminals observed after administration of NA preceded by malamide (or of α methyl NA) to non reserpinized animals on the other hand showed mainly a more or less marked increase in the intervaricose segments which thus looked more prominent and also thicker than normal (see above) The terminals presented this smooth appearance even at very low fluorescence intensities (Fig 26 42*f*)

As the fluorescence disappeared however the varicosities became slightly more marked although not nearly as prominent as in the normal iris (Fig 27)

The non terminal axons showed a remarkably increased intensity higher than in the terminals which made them easy to observe (Fig 19) They were uniformly thick and fluorescent in the same way as in normal iris and they appeared at the same time as the terminals No obvious difference in disappearance rate was observed between the terminals and the non terminal axons

(+) NA (0 02 0 1 and 2 mg/kg i v —10 rats) preceded by malamide gave about the same fluorescence intensity as the same dose of (−) NA preceded by malamide or (−) α methyl NA The highest dose gave a very strong fluorescence with the same appearance as after the laevo forms

DA (or α-methyl-DA)

DA (5 mg/kg i v —4 rats—or 10^{-5} *M* initial concentration intraocularly—3 rats) given 5 min before death produced no restitution of specific fluorescence immediately after death but weakly fluorescent fibres could be found 15 min post mortem Local application of the same concentration 5 min post mortem gave rise to a weak to moderate fluorescence 15 min after administration

If the animals were pretreated with nialamide (100 mg/kg) 2 h before the administration of DA (0 05 to 5 mg/kg i v 2 to 10 mg/kg i p or s c or 10^{-} to 10^{-5} *M* initial concentration intraocularly—Table 16) it was possible to observe a weak specific fluorescence 15 min after 0 2 mg/kg i v or 10^{-7} *M* intraocularly while 5 mg/kg i v or 10^{-5} *M* intraocularly gave almost the same fluorescence intensity as in the normal iris As early as 5 min after the administration of 1 mg/kg i v the fluorescence had assumed the same moderate intensity as after 15 min The restituted fluorescence disappeared slowly and was still present—at a low intensity—6 h after administration

TABLE 16 *The intensity of the restituted fluorescence of the adrenergic nerves after the administration of DA preceded by nialamide[1] to reserpinized animals*

DA i v

Dose (mg/kg)	Time before death (h)					
	0 1	0 5	1	2	4	6
0 05		(+) (2)				
0 2		+ (6)				
1	+ + (4)	+ + (12) (8)	+ + (4)	+(+) (4)	+ (?) (?)*	+ (4)
5		+ + +[a] (5)				

Increased background fluorescence

DA intraocularly

Initial concentration (M)	15 min before death
10	+ ( ) (4)
10	+ + (5) (4)
10	+ + + (4)

DA i p or s c

Dose (mg/kg)	1 h before death
	+ + (5)
10	+ + + (10)

α Methyl DA (1 mg/kg i v —6 rats) caused about the same fluorescence as an identical dose of DA preceded by MAOI

The appearance of the fluorescence after DA (or α methyl DA) was something between the normal appearance and the appearance of the fluorescence after NA (or α methyl NA) The non terminal axons were relatively *pronounced and could be easily seen while the terminals showed* varicosities that were not as accentuated as in a normal iris but definitively more prominent than after NA (Fig 20 24 32 42 *d*) The colour of the fluorescence was judged to be the same as in normal irises The appearance and intensity of the fluorescence after DA were the same after depletion with 10 mg/kg reserpine 4 h before the DA as after 5 mg/kg 18 h before

DOPA DOPS 5 HT and 5 HTP

No restitution of the specific fluorescence occurred when L DOPA (1 mg/kg i v —3 rats—or 20 mg/kg i p —3 rats) was given to reserpinized animals If however the animals were also treated with nialamide (100 mg/kg) 2 h before L DOPA (0 2 to 5 mg/kg i v or 1 to 20 mg/kg i p — Table 17) then such a restitution was observed This restitution was weak 1 h after 1 mg/kg i p and 15 min after 0 2 mg/kg i v Twenty mg/kg i p and 5 mg/kg i v gave nearly the same fluorescence intensity as in a normal iris 5 min after 5 mg/kg i v the fluorescence was almost the same as after 15 min The highest doses gave rise to a slight increase in background

TABLE 17 *The intensity of the restituted fluorescence of the adrenergic nerves after the administration of L DOPA preceded by nialamide[1] to reserpinized animals and the effect of certain drugs on restitution*

Dose (mg/kg)	Time before death (h) 0 1	0 25	1	
i p				
1			+ (3)	
			+ + (4)	
20			+ + (+)[a] (6)	
i v				
0 2		(+) (3)		Even after desmethyl imipramine imipramine or chlorpromazine
1		+(+) (12)		
5	[a] (2)	+ + (+)[a] (8)		
		(3)		100 mg/kg NSD 1015 1 h before L DOPA

[a] Increased background fluorescence

fluorescence Ligation of the arteries to the kidneys liver and intestines did not influence the restitution of the specific fluorescence after intravenous injection to any greater extent

A weak to moderate restitution of fluorescence was observed al o after intraocular administrations (10^{-6} to 10^{-5} *M* initial concentration intra ocularly) if the animals were pretreated with nialamide (100 mg/kg)

It has been shown (see chapter IX) that desmethylimipramine prevents the restitution of fluorescence after moderate doses of DA (1 mg/kg i v) preceded by nialamide *Only a slight reduction in the fluorescence was* noted however following L DOPA preceded by desmethylimipramine

If the animals had received the DOPA decarboxylase inhibitor NSD 1015 (100 mg/kg) 1 h before L DOPA no restitution occurred even after nialamide treatment

The appearance of the restituted fluorescence after L-DOPA was the same as after DA (Fig 29)

Only after 20 mg/kg D DOPA i p preceded by nialamide was there a weak but definite restitution of fluorescence Intravenous (1 to 5 mg/kg) and intraocular (10^{-5} *M*) administrations have not so far given reliable evidence of restitution

Restitution of the specific fluorescence 1 h after α methyl DOPA (10 mg/kg i p or s c —8 rats) was very weak in some of the animals and completely absent in the others

DOPS (50 mg/kg i p —3 rats) preceded by nialamide caused on the other hand a weak fluorescence 1 h after its administration This fluorescence however was too weak to decide whether it resembled more that after NA or that after DA No restitution of fluorescence occurred if the animals (2 rats) were treated with NSD 1015 (100 mg/kg) 1 h before DOPS

1 h after the administration of 5 HT (20 to 100 mg/kg i p —10 rats) preceded by nialamide there were found weakly to very weakly fluorescent fibres with the same distribution as the adrenergic nerves The fluorescence however was too weak for its colour to be determined with any certainty The appearance of the restituted fluorescence was about the same after very low doses of α methyl NA or NA preceded by nialamide The terminals looked fairly smooth and the non terminal axons were more or less pro nounced 5 HTP (100 mg/kg i p —4 rats) preceded by nialamide gave about the same results as 5 HT 1 h after administration

DISCUSSION

The increased intensity of the specific fluorescence of the adrenergic nerves in the iris of normal animals following the administration of NA

or α-methyl NA is no doubt due to increased levels of CA. There is likewise convincing evidence that the restituted specific fluorescence appearing after the administration of NA or α-methyl NA in irises depleted of their NA by reserpine is due to the amines themselves, since neither the 3-O-methylated nor the deaminated metabolites formed from the administered CA give the fluorescence reaction and the histochemical criteria of the fluorescence method are fulfilled.

It has not, on the other hand, been possible as yet to decide histochemically whether the restituted fluorescence after treatment with DA (α-methyl DA) is due to DA or NA (α-methyl DA or α-methyl NA). In normal animals DA (α-methyl DA) is probably converted to NA (α-methyl NA). Since, however, the β-hydroxylation of DA, which takes place in the amine granules (KIRSHNER 1957, AXELROD and POTTER 1963 *b*) might be less efficient if DA were not taken up by the granules in reserpinized animals, the specific fluorescence in these animals could also be due to DA (α-methyl DA).

Since no fluorescence is observed in reserpinized animals after L-DOPA preceded by malamide when decarboxylation is inhibited by NSD 1015 (CARLSSON 1964), it must be assumed that the fluorescence after L-DOPA is due not to L-DOPA itself but probably to DA or NA. By the same token, the fluorescence after DOPS is probably due to NA rather than to the amino acid.

The restituted fluorescence is localized to fibres with the same general distribution as the adrenergic fibres in normal animals and no restitution was observed in the degenerating adrenergic *nerves or in any other type* of nerve fibres 48 h after cervical sympathectomy (MALMFORS and SACHS 1965*a*), which causes a complete disappearance of the *normal* fluorescence 12 to 36 h after operation. There is thus no doubt that the CA were localized to the adrenergic fibres, both in the *non-reserpinized animals* and in the reserpinized ones. It is likewise probable that the weak fluorescence after 5-HT and 5-HTP was localized to the adrenergic nerves and due to 5-HT.

The subcellular localization of the CA will be considered in the General Discussion.

It can be concluded that CA are easily and rapidly taken up and accumulated by the adrenergic nerves, while 5-HT is very poorly taken up. It is also clear that L-DOPA can penetrate into the nerves and be decarboxylated intraneuronally to DA, since it has been shown (see chapter IX) that desmethylimipramine prevents the restitution of the fluorescence after DA (1 mg/kg i.v.) preceded by malamide, while the fluorescence after L-DOPA (1 mg/kg i.v.) was only slightly reduced by this agent. Also a local adminis-

tration or an intravenous injection after ligation of the renal and intestinal arteries—which greatly reduce the extraneuronal decarboxylation—gave approximately the same results It is impossible to decide whether D DOPA penetrates the axon membrane less efficiently than L DOPA the poor restitution of fluorescence after D DOPA could be due to the well known stereo specificity of the decarboxylating enzyme The weak fluorescence observed after intraperitoneal injection could be due to contamination of D DOPA with L-DOPA

VI EFFECT OF MAO INHIBITION

It is well known that endogenous NA and DA are metabolized partly by MAO which has been localized to the nervous tissues Various different compounds have been found to inhibit the action of MAO It has been found that an effective MAO inhibition raises the amine levels in the brain of rat and mouse (CARLSSON and LINDQVIST 1962) Also in the case of the peripheral nervous system there is clear evidence (MUSCHOLL 1959 PLETSCHER GEY and ZELLER 1960 CROUT CREVELING and UDENFRIEND 1961) that the NA content may be increased in normal animals following the administration of MAOI MAO seems to be very important for the amine metabolism of adrenergic nerves (CARLSSON 1960) and seems to have a high activity in the rat since its inhibition is necessary for a clear histochemical or biochemical demonstration of the uptake and accumulation of exogenous NA and DA at least in reserpinized animals (ANDÉN CARLSSON and WALDECK 1963 HAMBERGER MALMFORS NORBERG and SACHS 1964) The effect of different MAOI on MAO in the peripheral nervous tissues can thus be studied in respect of their ability to potentiate the accumulation of exogenous CA as studied by the fluorescence method

By using several MAOI of quite different chemical structure it seems possible to exclude non specific effects

There is little doubt from histochemical studies that MAO is localized intraneuronally in at least the cell bodies of the adrenergic neurons (KOELLE and VALK 1954 NORBERG unpublished observations) but the fluorescence method offers a possibility of obtaining more definitive evidence on this for the whole neuron (see the General Discussion)

MAO is highly dependent on an adequate oxygen tension while the uptake accumulation mechanism seems to be much less sensitive in this respect (see chapter V) Anoxia can therefore be used for MAO inhibition

In this chapter the effects of nialamide in normal and reserpinized animals have been studied with the fluorescence method Several MAOI namely pargyline iproniazid pheniprazine harmaline harmine and nialamide have been tested in respect of their ability to potentiate the restituted fluorescence the latter two drugs in different doses at different times before the administration of CA and in respect of the duration of their effects The effects of anoxia have also been tested

Table 18 *Effect of nialamide on the NA content of adrenergic nerves in normal animals*

Dose (mg/kg)	Time before death (h)				
	1.5	3	5	8	24
100	+ + + (11)	+ + +(+) (6)	+ + + + ( )	+ + + + (4)	+ + +(+) (4)

Effect of nialamide in normal animals

When nialamide (100 mg/kg—Table 18) was given 1.5 to 24 h before death to untreated animals a slight but noticeable increase in fluorescence intensity was observed in the adrenergic nerves after 3 h and a strong increase after 5 to 8 h. No further increase was seen after 24 h. The varicosities and even more clearly the segments between them showed a stronger fluorescence than normally as did the non-terminal axons which could thus be easily observed 5 to 8 h after treatment (Fig. 21).

Effect of nialamide in reserpinized animals

When nialamide (100 mg/kg—Table 19) was given 2 to 18 h after reserpine (10 mg/kg) and 2 to 8 h before death a very weak specific fluorescence was observed in the terminals in parts of the iris where the background fluorescence was weak. A somewhat higher fluorescence was found in the large non-terminal axons. This restitution of the specific fluorescence in reserpinized animals by nialamide alone was relatively evident 5 to 8 h after nialamide treatment in the animals treated with reserpine 18 h

Table 19 *Effect of nialamide on the NA content of adrenergic nerves in reserpinized animals*

Reserpine (10 mg/kg i.p.) Time before nialamide (h)	Nialamide (100 mg/kg) Time before death (h)			
	2	3	5	8
2	− (5)			
4		− (2)	(+) (5)	
18		(+) (4)	(+) (4)	(+) (4)

previously, while no specific fluorescence at all could be found up to 3 h after nialamide treatment in the animals treated with reserpine 2 to 4 h previously.

Effect of different MAOI in potentiating the intraneuronal accumulation of NA

The possibility of examining the effects of different MAOI is based on the finding that an inhibition of MAO is necessary to be able to observe the uptake of exogenous NA in reserpinized animals (HAMBERGER, MALMFORS, NORBERG and SACHS 1964).

All the animals received reserpine (5 mg/kg) 16 to 20 h before administration of the MAOI and NA (0.02 to 0.1 mg/kg i.v.) was given 15 min before death.

When nialamide (100 mg/kg—Table 20) was given at different times before the administration of NA, an almost maximal effect on the MAO, as judged from the fluorescence intensity, was observed already after 30 min. Roughly the same effect was found up to at least 24 h after the administration of nialamide. This agrees very well with the biochemical data which have shown the same long lasting effects of nialamide (WALDECK, personal communication).

Nialamide seemed greatly to reduce the effect of MAO in the iris, since widely varying doses (20 to 500 mg/kg—Table 21) 3 h before the administration of NA all had much the same effect. A further support for this assumption is that the NA taken up and accumulated after nialamide (100 mg/kg) disappears spontaneously at much the same rate as corresponding amounts of α-methyl NA (see chapter V). 5 mg/kg nialamide had a weak inhibiting effect, much lower than 20 mg/kg.

TABLE 20. *Effect of varying intervals between nialamide and NA on the intensity of the restituted fluorescence of the adrenergic nerves in reserpinized animals*[2]

NA (mg/kg i.v.) 15 min before death	Nialamide (100 mg/kg) Time before NA (h)				
	0.5	2	4	6	24[a]
0.02	+ (3)	( ) (4)	+(+) (3)	+(+) (2)	+ (4)
0.1	(3)	+ + + (4)	+ + + (3)	+ + + (2)	+ + + (3)

[a] Reserpine (10 mg/kg i.p.) 4 h before nialamide.

TABLE 21. *Effect of varying doses of nialamide before NA on the intensity of the restituted fluorescence of the adrenergic nerves in reserpinized animals*[a]

NA (mg/kg i.v.) 15 *min* before death	Nialamide dose (mg/kg) 3 h before NA			
	0	20	100	500
0.02	− (4)	+(+) (4)	+(+) (3)	++ (3)
0.1	+ (3)	+++ (4)	+++ (4)	+++ (3)

In comparison with the maximal inhibiting effect of nialamide, pheniprazine (10 mg/kg i.v.—Table 22) 4 h before the administration of NA had about the same effect; pargyline (200 mg/kg i.p.—Table 22) 6 h before administration almost the same effect; iproniazid (100 mg/kg i.v.—Table 22) 12 to 17 h before administration slightly less effect; and harmaline (20 mg/kg i.p.—Table 22) 30 min before administration almost the same effect. It was somewhat difficult to see the restitution of the specific fluorescence in the irises of the harmaline treated animals, since harmaline has a very strong yellow autofluorescence. This, however, can be destroyed by u.v. illumination during a few seconds in the microscope or elsewhere. Such treatment had no effect at all on the specific fluorescence. No noticeable

TABLE 22. *Effect of certain other MAOI in potentiating the restitution of the fluorescence intensity of the adrenergic nerves in reserpinized animals*

	MAOI		NA (mg/kg i.v.)	
	Dose (mg/kg)	Time before NA (h)	0.02	0.1
			15 min before death	
Pheniprazine i.v.	10	4	+(+) (3)	+++ (4)
Pargyline i.p.	200	6	+ (3)	++ (3)
Iproniazid[a] i.v.	100	12–17	+ (3)	++ (4)
Harmaline i.p.	20	0.5	+ (2)	+++ (4)
Harmine i.p.	20	0.5	+(+) (2)	+++ (3)
Harmine i.v.	2	0.25	+(+) (2)	+++ (6)
			1 h before death	
		0.75		+ (4)
			2 h before death	
		0.25		− (4)

[a] Reserpine (10 mg/kg i.p.) 4 h before iproniazid

accumulation of harmaline was observed in any special structure at least not in the axons (There may have been a suggestion of such accumulation in the Schwann cells) Harmine (2 mg/kg i v or 20 mg/kg i p —Table 22) 15 or 30 min respectively before the administration of NA had about the same effect as nialamide Harmaline and harmine are known unlike the other MAOI mentioned to have a rapid but rather short lasting effect (PLETSCHER GEY and ZELLER 1960) This was confirmed when the spontaneous disappearance of the NA taken up and accumulated after harmine was compared with the disappearance of corresponding amounts of NA taken up and accumulated after nialamide It was found that the NA taken up and accumulated after harmine (2 mg/kg i v 15 min before the NA—Table 22) had almost completely disappeared 1 h after and completely disappeared 2 h after administration The NA taken up and accumulated after nialamide is present at least 6 to 8 h after administration

Effect of anoxia on the activity of MAO

As described above the specific fluorescence could be restituted post mortem if a sufficiently high dose of NA (5 mg/kg i p 0 1 mg/kg i v or 10^{-6} to 10^{-4} *M* initial concentration intraocularly) was given less than 15 min before death A local administration of NA (10^{-6} to 10^{-4} *M* initial concentration intraocularly) shortly post mortem (5 min) could also restitute the fluorescence This means that the uptake and accumulation mechanism functions even at low oxygen tensions when the oxidative deamination by MAO is reduced and that NA is present extraneuronally at least 5 min after the administration of the large doses

DISCUSSION

From the results in this chapter it seems clear that MAO is extremely important for the metabolism of the intra axonal amines

Since the inhibition of MAO by e g nialamide caused a significant increase in the amount of NA in the adrenergic nerves in normal animals MAO may regulate the transmitter content by metabolizing the excess This increase cannot be due merely to an inhibition of the spontaneous release of NA (AXELROD HERTTING and PATRICK 1961) caused by a ganglion blockade (HERTTING POTTER and AXELROD 1962) since decentralization had no noticeable effect on the content of NA (MALMFORS unpublished results)

From the experiments in this chapter and chapter V it is also obvious that the activity of MAO is very high in rat iris as there are no or only

very weak signs of accumulation of CA after quite high doses of CA unless MAO is effectively inhibited After treatment with a MAOI however a weak restitution could be found even after 10 to 50 times lower doses

MAO inhibition by malamide—as studied with the present method—seems to be almost complete as early as 30 min and up to 24 h after the administration of 100 mg/kg i p

There is little doubt that the effect of MAOI is due in fact to the inhibition of MAO since so many drugs with quite different chemical structures have the same action

There is strong evidence that the MAO responsible for the intra axonal amine metabolism is in fact localized intra axonally (see the General Discussion)

VII DEGENERATION OF THE ADRENERGIC NERVES AS REVEALED BY THE FLUORESCENCE METHOD

It is generally accepted that the adrenergic nerves lose their NA content some time after having been separated from the cell body by axotomy. The disappearance has been regarded as the result of simple degeneration. Its time course in different species has been described by several authors (KIRPEKAR, CERVONI and FURCHGOTT 1962; TRENDELENBURG 1963a and b; BENMILOUD and EULER 1963).

The development of the cocaine-like component in the degeneration supersensitivity has been correlated to the disappearance of the transmitter, but no adequate reason for this correlation has been suggested. Using the iris technique, the adrenergic nerves have been studied during the course of degeneration following axotomy in respect of the intraneuronal distribution and disappearance of the transmitter, the loss of amine uptake-storage mechanisms and the effects of reserpine, bretylium and a potent MAOI (nialamide). The details of these investigations have been published separately (MALMFORS and SACHS 1965a) and there will be given here only a brief review in illustration of the iris technique.

In agreement with BENMILOUD and EULER (1963), the amount of transmitter remained more or less unchanged up to 8 h after axotomy and then disappeared during the next 16 h. This slow biochemical disappearance, however, did not reflect at all the course of events in the individual nerves from which the transmitter seemed to disappear completely within the space of 1 to 2 h. To judge from the overall picture of a whole mount of iris during degeneration (Fig. 36) and from the percentage disappearance of the normally fluorescent fibres at different times, disappearance starts at varying times after axotomy in the individual systems of terminals. The morphological picture suggests that the transmitter disappears uniformly and at the same time from entire systems of branching terminals belonging to one and the same neuron, since systems of intact terminals—like those thought to be derived from the axons of individual adrenergic neurons—were to be found during the degeneration (Fig. 35). During disappearance the terminals assumed a special appearance with very pronounced varicosities and very weak segments between. These varicosities became still

more pronounced after pretreatment with nialamide or nialamide and NA (Fig 37) If the animals were given reserpine shortly after the operation the fluorescence disappeared in about the same way as in animals with an intact innervation of the iris The fluorescence could then be restituted with NA preceded by nialamide (or with α methyl NA) in about the same per cent of terminals as those which showed normal fluorescence in non reserpinized animals at corresponding times of degeneration The restituted fluorescent terminals had the smooth appearance earlier described and the same distribution as the normal fluorescent terminals during degeneration It was concluded from these observations that the uptake storage mechanisms of the adrenergic nerves operate more or less unchanged for a certain time after axotomy and then deteriorate rapidly at about the same time as the transmitter disappears For the reasons given in a previous paper (MALMFORS and SACHS 1965a) it seems probable that the uptake accumulation mechanism of the axon membrane which is insensitive to reserpine disappears somewhat before the storage mechanism of the amine granules The disappearance of the axon membrane mechanism which may be due to degenerative changes in the membrane makes possible a passive leakage of the transmitter

These results thus clarify why the cocaine like component in the denervation supersensitivity which is believed to be due to the loss of the uptake mechanisms is developed simultaneously with the disappearance of NA

VIII INFLUENCE OF INCREASED ACTIVITY IN THE ADRENERGIC NERVES UPON THEIR CA LEVELS

It has been considered that increased activity in the adrenergic nerves produced e g by electric stimulation has no or very little effect on their NA level (EULER and HELLNER BJORKMAN 1955) due to effective mechanisms for keeping a constant level of transmitter There has later however been demonstrated a marked decrease in the NA content of vasoconstrictor nerves in cat following a quite moderate stimulation (KERNELL and SEDVALL 1964) It is probable that several different mechanisms are involved in maintaining a high transmitter content at increased activity e g a new formation of NA and a recapture of the released transmitter (STJARNE 1964)

This chapter will describe the effect of increased activity on the adrenergic nerves in the iris following inhibition of the NA synthesis by recently introduced inhibitors of the first rate limiting step in the NA biosynthesis (CARLSSON CORRODI and WALDECK 1963) Some preliminary data on this have already been published (MALMFORS 1964) The effect of increased activity in the nerves on the depletion of the endogenous NA by reserpine and on the exogenous NA (or the α methyl NA) taken up and accumulated in reserpinized animals has also been studied with the fluorescence method and the iris technique

Influence of increased nerve activity on the transmitter content of the adrenergic nerves after inhibition of the NA synthesis

After stimulation of the cervical sympathetic trunk in animals not treated with drugs there was a certain decrease in the fluorescence intensity of the terminals after stimulation for 20 min (20/sec) and a somewhat greater decrease after 40 min (20/sec – Table 23—Fig 38) The non terminal axons on the other hand showed a normal or sometimes even increased intensity The decrease in fluorescence intensity in the terminals was located mainly to the varicosities

When the right sympathetic trunk was stimulated for 40 min (20/sec) 30 min after the administration of some of the synthesis inhibitors (H22/54—6 rats—or H33/07—5 rats 500 mg/kg i p) the specific fluorescence in the right iris was significantly (Fig 41) weaker than that of the left intact iris

and that of the stimulated iris in an animal not treated with drugs The decrease in specific fluorescence was located to the terminals which in some areas had completely disappeared and in some others been strongly reduced In the weakly fluorescent fibres the varicosities had become relatively less pronounced than in a normal iris while the segments between them were only slightly affected giving the terminals a smooth appearance (Fig 42f) like that of the restituted fluorescent fibres after NA (or α methyl NA) Some terminals sometimes with the same appearance as a system of branching terminals from a single axon and the non terminal axons showed a practically unchanged fluorescence intensity

Influence of increased activity on depletion of the adrenergic transmitter by reserpine

When the right sympathetic trunk was stimulated for 20 min (20/sec—Table 23) 10 min after the administration of reserpine (5 mg/kg i v) there was observed a great reduction in the specific fluorescence in the terminals except sometimes for some more or less unchanged branching terminals (cf above) The fluorescent fibres showed a smooth appearance (cf above Fig 42f) contrary to that observed during depletion by reserpine without stimulation (Fig 14 15 42b) However the non terminal axons especially the large ones showed a practically unchanged fluorescence intensity In the left iris both the terminals and the non terminal axons showed an almost normal fluorescence intensity After 40 min of stimulation (Table 23) only some very faint terminals could be found in addition to the still more or

TABLE 23 *Effect of electric stimulation on endogenous NA*

Pretreatment of the animal	Time of stimulation (20 sec) 0 min: Stimulated iris	0 min: Non stimulated iris	40 min: Stimulated iris	40 min: Non stimulated iris
—	+(+) (6)	+ + + (6)	+ + (5)	+ + + (5)
Reserpine (5 mg/kg i v) 10 min before stimulation	+ (6)	+ + + (6)	(+) (6)	+ + (+) (6)
Nialamide (100 mg/kg) 4 h and reserpine (5 mg/kg i v) 10 min before stimulation	+ + (+) (5)	+ + +(+) (5)	+ + (5)	+ + +(+) (5)

VIII INFLUENCE OF INCREASED ACTIVITY IN THE ADRENERGIC NERVES UPON THEIR CA LEVELS

It has been considered that increased activity in the adrenergic nerves produced e g by electric stimulation has no or very little effect on their NA level (EULER and HELLNER BJORKMAN 1955) due to effective mechanisms for keeping a constant level of transmitter There has later however been demonstrated a marked decrease in the NA content of vasoconstrictor nerves in cat following a quite moderate stimulation (KERNELL and SEDVALL 1964) It is probable that several different mechanisms are involved in maintaining a high transmitter content at increased activity e g a new formation of NA and a recapture of the released transmitter (STJÄRNE 1964)

This chapter will describe the effect of increased activity on the adrenergic nerves in the iris following inhibition of the NA synthesis by recently introduced inhibitors of the first rate limiting step in the NA biosynthesis (CARLSSON CORRODI and WALDECK 1963) Some preliminary data on this have already been published (MALMFORS 1964) The effect of increased activity in the nerves on the depletion of the endogenous NA by reserpine and on the exogenous NA (or the α methyl NA) taken up and accumulated in reserpinized animals, has also been studied with the fluorescence method and the iris technique

Influence of increased nerve activity on the transmitter content of the adrenergic nerves after inhibition of the NA synthesis

After stimulation of the cervical sympathetic trunk in animals not treated with drugs there was a certain decrease in the fluorescence intensity of the terminals after stimulation for 20 min (20/sec) and a somewhat greater decrease after 40 min (20/sec—Table 23—Fig 38) The non terminal axons on the other hand showed a normal or sometimes even increased intensity The decrease in fluorescence intensity in the terminals was located mainly to the varicosities

When the right sympathetic trunk was stimulated for 40 min (20/sec) 30 min after the administration of some of the synthesis inhibitors (H22/54—6 rats—or H33/07—5 rats 500 mg/kg i p) the specific fluorescence in the right iris was significantly (Fig 41) weaker than that of the left intact iris

nerves in reserpinized animals seems to indicate a direct action on the amine granules by the nerve impulses that release the transmitter It is also possible however although less likely that the recapture mechanism (see the General Discussion) in the axon membrane is so effective that there is no net decrease in CA when the intraaxonal enzymatic destruction of CA is diminished On the other hand a small but definite decrease in the transmitter content is observed at stimulation after reserpine treatment if MAO is inhibited by nialamide This however could be due to other factors (see below)

Reserpine does not seem to interfere primarily with the biosynthesis (PAULSEN and HESS 1963) or the recapture of NA (see chapter IX) so that the rapid depletion on stimulation after pretreatment with reserpine can hardly be explained by impairment of these mechanisms (cf ROSELL and SEDVALL 1962) The main action of reserpine seems to be an inhibition of the NA uptake into the amine granules (see CARLSSON 1965*a* and the General Discussion)

NA seems to have to be located in the amine granules to avoid destruction by MAO Since nerve stimulation leads to an increased disappearance of NA from the amine granules the rapid depletion by nerve stimulation after reserpine seems to be due to a rapid establishing of the reserpine blockade of the amine granules leading to an increased breakdown by MAO of the transmitter (The latter being either directly released into the extragranular cytoplasm or recaptured from the outside of the nerves into the cytoplasm by the membrane mechanism after release from the nerve) This is further supported by the finding that MAO inhibition almost completely prevents the rapid disappearance of NA at stimulation preceded by reserpine treatment This prevention may also be due to extragranularly located NA interfering with the inhibition of the amine granules by reserpine The morphological picture of the stimulated nerves speaks however in favour of a higher proportion of NA extragranularly in their cytoplasm than in that of unstimulated nerves after the same treatment This may indicate an inhibition of uptake into the amine granules of NA released from the granules by nerve impulses

The rapid depletion by nerve stimulation after reserpine treatment could also partly be due to an indirect synthesis inhibition since freshly formed DA cannot readily be taken up into the granules and probably not β hydroxylated to NA (KIRSHNER RORIE and KAMIN 1963)

IX. EFFECT OF VARIOUS DRUGS ON THE UPTAKE, ACCUMULATION AND STORAGE OF EXOGENOUS NA (α-METHYL-NA) IN THE ADRENERGIC NERVES

Several drugs have been tested in respect of their ability to interfere with the uptake, accumulation and storage of labelled exogenous NA in the tissues of non-reserpinized animals (AXELROD, WHITBY and HERTTING 1961; HERTTING, AXELROD and PATRICK 1961, 1962; HERTTING, AXELROD and WHITBY 1961; MUSCHOLL 1961; AXELROD, HERTTING and POTTER 1962; IVERSEN 1964). However, as there seem to exist at least two different uptake-concentration mechanisms in the intact adrenergic nerves (see the General Discussion), it is difficult to decide the mode and site of these different drugs. Some of these have been shown to inhibit the uptake of amines into amine granules *in vitro* (CARLSSON, HILLARP and WALDECK 1963). On the other hand there are several reasons for believing that e.g. cocaine inhibits the uptake of exogenous NA by tissues in a quite different way, since cocaine has a very little effect on the uptake of amines into amine granules (CARLSSON, HILLARP and WALDECK 1963). Furthermore it has been shown in a previous preliminary paper (HILLARP and MALMFORS 1964) that cocaine and certain other drugs inhibit an uptake-concentration mechanism that is distinct from that in the amine granules and which is probably located to the cell membrane of the entire adrenergic neuron. Reserpine, however, seemed to have no or very little effect on this mechanism (see below). It is therefore possible to study the effect of drugs on the uptake, accumulation and storage of exogenous CA in the adrenergic nerves even in reserpinized animals where the amines are not taken up by the amine granules.

In the experiments reported in this chapter the fluorescence method and the iris technique were used partly for a preliminary screening among drugs that are supposed to have a cocaine-like effect and partly for further investigations on the uptake-concentration mechanism that is probably located to the axon membrane and on its inhibition. Most of the experiments were performed in reserpinized animals with NA preceded by nialamide or with α-methyl-NA, but non-reserpinized animals and α-methyl-DA or DA preceded by nialamide were also used.

All drugs (see Table 24) have been tested in respect of their ability to

prevent the uptake and accumulation of CA and some of them (see Table 24) were also tested in respect of their ability to interfere with the spontaneous disappearance of the CA taken up in the adrenergic nerves

Inhibition of the uptake and accumulation of exogenous CA in the adrenergic nerves

NA (or α methyl NA) to non reserpinized animals

When NA (0 1 mg/kg i v) preceded by nialamide or α methyl NA (0 1 mg/kg i v) was given to non reserpinized animals 15 min before death there was a slight but evident increase in the fluorescence intensity of the adrenergic nerves—especially in the non terminal axons—as compared with the controls (see chapter V) This increase did not appear if the animals had been pretreated with e g desmethylimipramine (25 mg/kg i p —6 rats) or protriptyline (25 mg/kg i p —6 rats) 30 min before the administration of NA (or α methyl NA half of the animals) and killed 15 min after This was most apparent in the non terminal axons where the fluorescence was as weak as in the control animals

NA (or α methyl NA) to reserpinized animals

NA (or α methyl NA—half of the animals) was usually given 30 min after the administration of the drug tested and the animals killed 15 min later The effect of the drugs tested in preventing the restitution of fluorescence by NA preceded by nialamide or by α methyl NA (see chapter V) varied considerably between the drugs By using different doses of NA (or α methyl NA) and of the tested drug it was possible to distinguish between drugs with a strong (protriptyline desmethylimipramine tyramine preceded by MAOI and amphetamine) moderate (metaraminol imipramine Ro4 6011 nortriptyline chlorpromazine promazine and cocaine) or weak (amitriptyline bretylium guanethidine chlorprothixene tetrabenazine) inhibitory effect while still others (trimeprimine levomepromazine ouabain phenoxybenzamine haloperidol reserpine prometazine chlorphenıramine and tripelenamine) seemed to have no or only very little effect

As seen from Table 24 protriptyline is the most effective inhibitor (cf Stone Porter Stavorski Ludden and Totaro 1964 Carlsson and Waldeck 1965b) This drug completely prevented the fluorescence restitution after 0 1 mg/kg NA (α methyl NA) when given in a dose of 10 mg/kg and almost completely in a dose of 2 mg/kg Desmethylimipramine which given 30 min before the administration of NA (α methyl NA) is almost as potent as protriptyline showed a strong inhibitory effect also when given

2 or 6 h before in the same dose Imipramine and chlorpromazine were also tested and found to have an inhibitory effect 2 h after administration

No evident differences were observed between inhibition of the fluorescence restitution after NA preceded by nialamide or that after α methyl NA except in the case of tyramine presumably because tyramine is a very good substrate for MAO But when the tyramine before α methyl NA was preceded by nialamide there was no longer any clear difference

DA (or α methyl DA) to reserpinized animals

When desmethylimipramine (25 to 50 mg/kg i p) was administered 30 min before DA (1 mg/kg i v) preceded by nialamide or before α methyl DA (1 mg/kg i v) there was no restitution of the specific fluorescence This was in contrast to the case with animals not treated with desmethylimipramine before DA or α methyl DA (see chapter V)

Depletion of exogenous NA (α methyl NA) from the adrenergic nerves in reserpinized animals

(See Table 24)

Some of the drugs tested were given 15 min after the administration of NA preceded by nialamide (or of α methyl NA—half of the animals) to reserpinized animals which were killed at various times after administration It was found that the drugs which have previously been shown to inhibit the uptake of exogenous NA (or α methyl NA) in reserpinized animals (tyramine metaraminol imipramine desmethylimipramine protriptyline bretylium cocaine and guanethidine) accelerate the disappearance of the restituted fluorescence reserpine however which did not inhibit this uptake of exogenous NA (or α methyl NA) by the mechanism probably located to the axon membrane had no significant influence on the disappearance Tyramine preceded by nialamide seemed to be the most effective depleting agent and metaraminol was almost as effective Some of the very weak fluorescence of the nerves after metaraminol may be due to metaraminol itself which under certain circumstances can give the fluorescence reaction (SACHS 1965) The depleting effect of the rest of the drugs seemed to be related to their previously demonstrated inhibitory effect Protriptyline and desmethylimipramine were thus extremely effective but not as effective as tyramine and metaraminol No or very little of the restituted fluorescence remained 45 min after the latter drugs while a weak but clearly noticeable fluorescence was found at the same interval after the former drugs and a very weak restituted fluorescence sometimes still remained 1 h later (see Fig 45) There was no definite difference in the loss of the resti

TABLE 24 *Influence of certain drugs on the restituted fluorescence after α methyl NA or NA preceded by nialamide in reserpinised animals*

rug te ted p unless otherwise stated)	Dose (mg/kg)	Time after the tested drug (h)	NA (α methyl NA) i v 15 min before death Dose (mg/kg) 0 0.	0 01	0 1 mg/kg NA (α methyl NA) i v 15 min before the tested drug Time before death (h) 0 5	1	2	4	8
yramine	2	0 25		+ (+)(4)					
	10	0 5		(+)(6)	+ (8)	(+)(8)	− (4)		
	50	0 25			(+)(6)	(+)(4)			
letaraminol	10	0 5	(+)(4)	+ (8)	+ (4)	+ (4)	+ (4)	(+)( )	
amphetamine	10	0 5	− (4)	(+) (8)					
m pramine	10	0 5	− (6)	+ (8)					
	10	2	(+)(4)	+ (+)(4)					
	50	0 5		− ( )	+ +(+)( )	+ + ( )	+ (4)	− (4)	
Desmethyl mi pramine	2	0		+ (4)					
	10	0		− (8)					
	10	2	− (4)	(+) (6)					
	10	6	− ( )	(2)					
					+ + (6)	+(+)(8)	(+)(8)	− (6)	
Trimeprim ne	50	0 5	− (6)	+ + + (4)					
Ro4 6011	10	0 5	(+)(6)	+ (4)					
	0	0 5	− (4)	(−) (4)					
A itriptyline	10	0	(−)(4)	− − ( )					
	0	0	− (6)	(−) ( )					
Nortriptyline	10	0	(−)(4)	− (4)					
	50	0 5	− ( )	(+) (4)					
Protriptyl ne	0 5	0 5		− ( ) (4)					
		0		(+) (4)					
	10	0 5		− (4)					
					− −(6) −	(8)	(+)(8) −	(4)	
P mazin	10	0 5	( )(4)						
	0	0	− (4)	+ (4)					
Chlorpromazine	10	0	( )(4)	− (6)					
	10		− (4)	− (4)					
	0	0	− (4)	(−) (4)		( )	(4) −	( )	
L vomepro mazine	50	0 5	− (4)	− (4)					
Chlorprothix ne	10	0 5	( )(4)	− (4)					
	50	0	− (6)						
B tylion	10	1	(4)	−( )(4)					
	0	1	( )(4)	(−) (4)		− ( )( )	( )(4)	(4)	− (
(a n	0	0 5	(4)	− (6)		+ − (4) −	(4)	(−)(4)	− (
(uan th line	10	0 5	+ (4)	−(−) (4)					
	30	0 5	− (4)	+ (4)		+( ) (4) −	(4)		

TABLE 24 (*Continued*)

Drug tested (i p unless otherwise stated)	Dose (mg/kg)	Time after the tested drug (h)	NA (α methyl NA) i v 15 min before death, Dose (mg/kg) 0.02	0.01	0.1 mg/kg NA (α methyl NA) i v 15 min before the tested drug, Time before death (h) 0.5	1	2	4	8
Ouabain i v	10	0.25	+ (6)						
Phenoxybenzamine i p	50	0.5	+ (6)	+ + + (2)					
i v	10	0.5	+ (6)						
Haloperidol	25	0.5	+ (4)	+ + − (2)					
Reserpine i v	10	0.25	− (6)	− − − (2)		+ + + (4)	+ + (4)	+(+)(_)	
Tetrabenazine	50	0.5	(−)(2)	− + (2)					
Prometazine	50	0.5	− (6)						
Chlorphenira mine	10	0.5	− (6)						
Tripelennamine	10	0.5	− (6)						
—			− (8)	− − (8)	− (8)	− − (8)	+ + (8)	+( )(8)	(+

tuted fluorescence after NA preceded by nialamide or after α methyl NA except in the case of tyramine presumably due here too to tyramine being a good substrate for MAO When the tyramine before α methyl NA was preceded by nialamide there was no longer any clear difference

DISCUSSION

The present results concerning the inhibition of the uptake of exogenous NA by tissues with the use of reserpinized animals are for the most part in good agreement with previous investigations on the uptake of H^3 NA by tissues made in non reserpinized animals (AXELROD WHITBY and HERTTING 1961 HERTTING AXELROD and WHITBY 1961 AXELROD HERTTING and POTTER 1962 IVERSEN 1964) Moreover the fluorescence method combined with the iris technique has given similar results also in non reserpinized animals In some cases (see below) however the new approach gave different results due to the fact that the animals had been pretreated with reserpine This difference between reserpinized and non reserpinized animals has recently been confirmed by CARLSSON and WALDECK (1965*b*) They also reported about the same relative inhibitory effect of many drugs used in this work in preventing the uptake of tritium labelled metaraminol in

reserpinized animals. This means in all probability that the inhibitory effect studied is localized to an uptake concentration mechanism distinct from that in the amine granules, the latter having already been effectively inhibited by reserpine.

It is possible that the inhibitory effect of the drugs tested works in different ways, as they differ in chemical composition and have various effects on the adrenergic nerves in non-reserpinized animals. The sympathomimetic amines (tyramine, metaraminol, amphetamine) for instance, are taken up by the adrenergic nerves (CARLSSON and WALDECK 1965a and b; SHORE, BUSFIELD and ALPERS 1964) and amine granules (ANDÉN 1964) and cause a depletion of the endogenous NA. Such a depletion is achieved also by guanethidine, which is likewise taken up by the nerves (CHANG, BRODIE and COSTA 1964). The heterocyclic compounds (imipramine, desmethylimipramine, Ro4-6011, amitriptyline, nortriptyline, protriptyline, promazine, chlorpromazine and chlorprothixene) on the other hand, have no significant depleting action on the endogenous NA (see CARLSSON 1965b) and may perhaps not be taken up and accumulated by the adrenergic nerves.

A fairly slight variation was observed in the effects of the sympathomimetic amines used, while the effects of the other drugs varied considerably. By comparing the effects of the different heterocyclic amines in relation to their chemical structure, it was possible to get some idea of which parts of the molecule are of importance for their inhibitory effect. The side chain seems to be the most important part of the molecule in this respect, as changes in the rings have very little effect, while changes in the side chain sometimes greatly alter the inhibitory effect. Thus drugs such as promazine, chlorpromazine, Ro4-6011 and imipramine, which have an identical side chain but somewhat different rings, have a roughly identical effect. Compounds, however, with quite different numbers and configurations of rings, but with the same side chain as tripelenamine and chlorpheniramine, had no inhibitory effect.

The linkage between the side chain and the middle ring seems to be of little importance when comparing e.g. imipramine and amitriptyline, but very important in the case of protriptyline.

Monomethyl derivatives are definitely more effective than dimethyl derivatives in preventing the uptake of NA, while a methyl group on the middle carbon atom in the side chain, as in trimeprimine and levomepromazine, seems completely to abolish the inhibitory effect (cf. BICKEL and BRODIE 1964). Further work, however, is needed before definite knowledge can be acquired as to the cause of this phenomenon. Evidence has been

published for the dimethyl derivatives being demethylated in the body (BICKEL, SULSER and BRODIE 1963).

In addition to the drugs already mentioned as having no inhibitory effect, it has been found that a further high dose of reserpine or a high dose of ouabain, phenoxybenzamine, haloperidol or prometazine has no or very little *inhibitory effect on the NA uptake into the adrenergic nerves of* reserpinized animals. In the case of reserpine this further confirms the opinion that the uptake concentration mechanism probably located in the axon membrane is insensitive to reserpine. In the case of ouabain the present findings agree with HERTTING, AXELROD and WHITBY (1961) but are in disagreement with the findings of DENGLER, MICHAELSON, SPIEGEL and TITUS (1962). Phenoxybenzamine has been found by several authors to inhibit the uptake of exogenous NA into storage sites (HERTTING, AXELROD and WHITBY 1961, THOENEN, HÜRLIMANN and HAEFELY 1963, ANDÉN, CORRODI, ETTLES, GUSTAFSSON and PERSSON 1964). This could not be demonstrated in the present paper. It is therefore possible that the previously observed effect of phenoxybenzamine is due mainly to the inhibition of the uptake concentration mechanism of the amine granules that has been observed *in vitro* with the use of isolated amine granules (CARLSSON, HILLARP and WALDECK 1963, EULER, STJÄRNE and LISHAJKO 1964).

Since it is possible to inhibit the uptake of DA (α-methyl DA) by the adrenergic nerves with some of the drugs tested, it is highly probable that DA is taken up in the adrenergic nerves by the same mechanism as NA (α-methyl NA).

From the experiments reported in chapter V it is obvious that either the passage out and in through the cell membrane of the NA (α-methyl NA) taken up in the adrenergic nerves of reserpinized animals is rather slow or else the recapture of the released CA is very effective, as the CA stay for a very long time (8 h). *From the present results* it is obvious that there is a recapture of NA by the uptake accumulation mechanism in the axon membrane, as the NA (α-methyl NA) accumulated extragranularly disappeared more rapidly after the uptake mechanism had been inhibited with e.g. desmethylimipramine. However, none of the heterocyclic drugs seems completely to inhibit the recapture through the cell membrane, probably not even protriptyline in high doses, since the depleting effect is related to the inhibitory effect. It is therefore possible that the passage rate of NA (α-methyl NA) out and in through the cell membrane is somewhat faster than that of the most rapid disappearance observed. The depleting effect of the sympathomimetic amines, however, is greater than what would be suspected from their inhibitory effect and greater than that of the hetero-

cyclic agents. At low NA concentrations this difference does not exist (CARLSSON 1965b).

The insensitivity to reserpine of the uptake concentration mechanism in the axon membrane is further supported by the finding that a second dose of reserpine has no effect on the spontaneous disappearance of the NA (α-methyl-NA) taken up extragranularly in the cytoplasm of the adrenergic nerves of reserpinized animals.

Bretylium showed no inhibitory effect on the disappearance of CA that has on some occasions been observed in non-reserpinized animals (HERTTING, AXELROD and PATRICK 1962; EULER and BENMILOUD 1963).

GENERAL DISCUSSION

The method used

The application of the fluorescence method of FALCK and HILLARP to studies on the adrenergic nerves compares favourably with the biochemical methods with or without the use of labelled amines in that it is possible directly to study the phenomena at cellular and subcellular levels, even with rather low concentrations of monoamines. The method consequently reveals the events in different parts of each separate adrenergic neuron; it has revealed certain fundamental facts on the uptake-concentration mechanisms in the adrenergic neurons and on the release of the adrenergic transmitter, facts not within the comprehension of biochemical techniques. It also reflects more adequately the events in the whole individual adrenergic neuron than the biochemical methods, which reveal an average picture of the events in several neurons. This difference between the two approaches became most apparent in studies on the degeneration of the adrenergic nerves and in the stimulation experiments.

Direct observations are facilitated by the specificity of the fluorescence method, which reveals of the compounds used only NA (α-methyl-NA), DA (α-methyl-DA), DOPA (α-methyl-DOPA), DOPS, 5-HT and 5-HTP and not their 3-O-methylated and/or deaminated metabolites (CORRODI and HILLARP 1963, 1964). It is consequently possible directly to observe the eventual neuronal uptake of NA or related amines independently of metabolites.

The fluorescence intensity in the range of that in a normal animal permits a fairly good semi-quantitative estimation, subjectively, of the amine concentrations in various parts of the neuron. It is true, however, that the fluorescence method gives no quantitative data due to the absence of any objective methods for determination of the fluorescence intensity, which does not seem to be directly correlated to the NA concentration in all ranges (RITZÉN, personal communication). In combination with other determinations, however, NORBERG and HAMBERGER (1964) have estimated the NA concentrations in different parts of the adrenergic neuron (see chapter III) in a normal animal. From these values and the comparison between the available biochemical data and the fluorescence intensity in corresponding

experiments it can be suggested that the lowest NA concentration which can be detected in the nerves under optimal conditions for the fluorescence method is about 10 to 100 μg/g wet wt If it is assumed that the nerves in the iris amount as in the spleen to not more than 1/1 000 to 1/10 000 of the organ weight (EULER 1956 p 136) and contain all the NA in the iris the specific fluorescence can be observed when the concentration of NA in the rat iris is as low as about 1 per cent of the normal value (this is about 1 μg/g wet wt HAGGENDAL and DAHLSTROM 1965 b) With irregular distributions of the amine within and among the nerves fluorescence can be detected at even lower total amounts of NA in the organ as a whole

By comparing the experimental irises with one another and with normal ones treated under the same histochemical conditions by using varying doses and intervals—especially of NA—and by taking into consideration the subcellular localization as shown by the morphological picture it has been possible to obtain reproducible semi quantitative data in this work Due to the high fluorescence intensity in the normal iris it is easier to observe and evaluate variations in the CA concentration when it is below than when it is above the normal value Especially when the fluorescence is weak to moderate the fluorescence method seems to give constantly reproducible results But it is also possible to observe a significant increase in the amine content as compared with normal since the terminals then—after e g MAOI or NA preceded by malamide—seem to have a slightly broader appearance than usual due to the strongly fluorescent intervaricose segments and as the non terminal axons have an increased fluorescence

The use of whole mounts of iris for the fluorescence method which simplifies the experimental conditions without any apparent disadvantage offers certain very important advantages which have brought to light information on the adrenergic nerves that it has been impossible to obtain from other tissues

a) The possibility of studying the adrenergic terminals and their non terminal axons along their whole length has furnished information as to the appearance and distribution of the axon and of the axon ramifications of an individual adrenergic neuron This information has furthermore been fundamental for the concept of systems of branching terminals as a morphological and functional unit which among other things is supported by the entire terminals probably all their varicosities being sites for the release of NA

b) *The possibility of treating the adrenergic nerves of the iris locally* by the administration of e g NA in relatively low concentrations in the anterior chamber of the eye has proved to be a good complement to the

systemic treatments as it makes it easier to estimate the NA concentration around the nerves avoids the systemic effects permits the use of one eye as a control of the effect of the intraocularly injected agent and allows comparison with *in vitro* studies (DENGLER SPIEGEL and TITUS 1961, DENGLER MICHAELSON SPIEGEL and TITUS 1962)

The usefulness and reliability of the present technique not only in morphological but also in physiological and pharmacological studies on the adrenergic nerves are demonstrated by the way in which results agree in principle with those of other studies using different techniques

The adrenergic ground plexus

The construction and functional organization of the autonomic innervation paparatus were described in a new way by HILLARP (1946 1949 1959) using the methylene blue technique With the fluorescence method of FALCK and HILLARP the distribution and construction of the adrenergic part of the autonomic ground plexus has now been investigated in a large number of organs (FALCK 1962 NORBERG and HAMBERGER 1964) It has been found that the adrenergic nerves form a network with for the most part two or more characteristically varicose fibres running together in each strand This plexus has been concluded to be the true adrenergic innervation structure and it has been supposed that the varicose fibres which contain most of the transmitter release the transmitter along their entire lengths and thus constitute the adrenergic terminals (see NORBERG 1965*b*) which together with the effector cells in their close proximity form a neuroeffector unit

The present investigations provide more direct proof of the organization of the innervation apparatus referred to above They demonstrate also the distribution of the axon from an individual adrenergic neuron within an effector organ which provides direct morphological evidence for the supposed functional organization of the autonomic innervation apparatus (HILLARP 1946 1949 1959)

The finding in the present study that isolated systems of branching varicose fibres remained in the iris after partial denervation is conclusive evidence that the adrenergic ground plexus consists of separate fibres from individual adrenergic neurons in agreement with the neuron doctrine as formulated by WALDEYER (1891) It refutes however the theories proposed for a syncytial organization of the autonomic innervation structure and/or a syncytial continuity between the nerves and the effector cells (for references see HILLARP 1946 1960) There is evidence moreover that such systems of branching terminals are also functional units since such forms

tions can be found more or less intact after stimulation in combination with inhibitors of the NA biosynthesis or reserpine—probably due to inadequate stimulation—while the fluorescence of most of the fibres is greatly reduced

The demonstration in rat iris of the distribution and appearance of the axon of an individual adrenergic neuron strongly supports and directly shows the correctness of the morphological explanation of the convergence principle in the physiology of the autonomic innervation apparatus as presented by HILLARP in 1959 when he stated The innervation structure *consists of the autonomic ground plexus within which each terminal axon* ramification has a certain extension and in its course innervates a certain number of cells or cell complexes forming a neuroeffector unit To each unit however several postganglionic neurons converge the terminal axon ramifications of which run within the same strands of the ground plexus By the overlap thus present in the innervation structure the response of the effector system may be modified by both temporal and spatial summation It also shows that the neuroeffector units belonging to one and the same neuron can be quite far away from each other in the effector organ which thus explains the diffuse character of the responses sometimes obtained on stimulation of only a small fraction of the nerves supplying a smooth muscle (LANGLEY 1904)

A very important assumption for the validity of HILLARP's concept of the functional organization of the autonomic innervation apparatus is that the transmitter really can be released along the entire length of the axon ramifications which form the ground plexus i e the varicose fibres That the transmitter must be stored in a structure of considerable length was assumed by HILLARP as the amounts of transmitter were calculated to be very large in the final parts of the adrenergic nerves He also proposed these structures as the sites for the release of transmitter in their entire length Since the fluorescence method has now made it possible to show that all the varicosities along the axon ramifications in the ground plexus are in all probability the sites of transmitter release HILLARP's concept of the construction and functional organization of the autonomic innervation apparatus offers a very reliable and adequate interpretation

The name terminal for that part of the axon ramifications which forms the ground plexus is likewise adequate since the latter is both morphologically and physiologically the presynaptic structure It is opposed to the purely morphological term nerve ending (RICHARDSON 1962 1964) which seems to include only the true ending of the axon ramifications which must play a minor role in the transmission between the adrenergic nerves and their effector cell

The subcellular localization of CA in the adrenergic nerves normally and under different experimental conditions

It has been found that the NA in normal adrenergic nerves is located in subcellular particles in both the non terminal axons (Euler and Hillarp 1956, Euler 1958) and in the terminals in different organs (Potter and Axelrod 1962, 1963c, Stjärne 1964, Euler and Lishajko 1965). In these investigations which were performed with differential centrifugation various amounts of the total NA were found in the particle fraction and most of the rest in the supernatant (Weil-Malherbe and Bone 1961, Michaelson, Richardson, Snyder and Titus 1964, Schümann, Schmidt and Philippu 1964, Snyder, Michaelson and Musacchio 1964 and Stjärne 1964). From such experiments it has been concluded that about half of the NA content in the terminals and a smaller proportion in the non terminal axons of the adrenergic neurons is bound to subcellular particles—the amine granules—while the rest of the intraneuronal NA is located extragranularly being either free in the cytoplasm or attached to some intraaxonal structure (Euler and Lishajko 1965).

The fluorescence method cannot furnish direct information as to the subcellular distribution of NA in the adrenergic nerves but in combination with drugs it has been shown to be probable that most of the NA is bound to the amine granules and that relatively small amounts are located extragranularly in normal nerves. This assumption is supported by the following findings

a) There is a good correlation between the concentration of NA and the number of granules or vesicles in various parts of the adrenergic neuron. In the weakly fluorescent non terminal axons electron microscopy has revealed few granules or vesicles while the strongly fluorescent varicosities have been found to contain a great number (Lever 1958, 1961, 1963, De Robertis 1962, 1964, Richardson 1962, 1964, Nilsson 1964).

b) In order to be able to demonstrate an accumulation of NA in the adrenergic nerves after low doses of NA in reserpinized animals—in which the NA uptake into the amine granules is completely blocked (Carlsson 1965a)—it is necessary to pretreat the animals with a potent MAOI (see above Andén, Carlsson and Waldeck 1963) which indicates that NA cannot exist extragranularly to any major extent in reserpinized animals unless it is protected from destruction by MAO.

c) Even quite high doses of NA (0.1 to 0.2 mg/kg i.v.) caused no visible increase in the weak fluorescence in the non terminal axons of non reserpinized animals unless NA was preceded by nialamide. This indicates that

NA cannot exist extragranularly to any major extent even in the non terminal axons of normal animals and also that the uptake accumulation of NA into the amine granules is rather limited in this case

In reserpinized animals on the other hand the NA (or α methyl NA) taken up seems to be located extragranularly as indicated by the fluorescence method The amounts of NA (or α methyl NA) taken up in different parts of the adrenergic nerves are not correlated to the number of granules or vesicles but rather to the diameter of the fibres The large non terminal axons thus showed the highest fluorescence intensity while the varicosities were much less pronounced than normal giving the terminals a smooth appearance Furthermore as already pointed out reserpine inhibits the uptake of NA into the amine granules

As the fluorescence method does not differentiate between DA and NA it is impossible directly to decide whether the specific fluorescence in reserpinized animals after DA (α methyl DA) is derived from DA (α methyl DA) or NA (α methyl NA) According to KIRSHNER ROBIE and KAMIN (1963) reserpine inhibits conversion of DA to NA in adrenomedullary granules But preliminary biochemical data indicate NA formation from DA in reserpinized animals (CORRODI and MALMFORS to be published) The intraaxonal localization of the fluorescence within the terminals after DA however is different from that after NA insofar as the varicosities are more pronounced after DA than after NA although still less pronounced than normal The non terminal axons were relatively pronounced after DA as after NA It is impossible at present to give a clear explanation for this difference Since however there was no restitution of the fluorescence without MAO inhibition there seems to be no uptake of CA into the amine granules in either case

Uptake and release of monoamines

Earlier investigations (HAMBERGER MALMFORS NORBERG and SACHS 1964 HILLARP and MALMFORS 1964 NORBERG 1965a) and the present study furnish conclusive direct evidence that the adrenergic nerves can take up and accumulate exogenous monoamines This is in agreement with the conclusions drawn by several other authors using other indirect methods (AXELROD WEIL MALHERBE and TOMCHICK 1959 MUSCHOLL 1960 STROMBLAD and NICKERSON 1961 ANDEN CARLSSON and WALDECK 1963) In addition to the uptake mechanism of the amine granules which has been studied *in vivo* (BERTLER HILLARP and ROSENGREN 1961) and extensively *in vitro*

(CARLSSON, HILLARP and WALDECK 1962, 1963; KIRSHNER 1962 a and b; EULER and LISHAJKO 1963 a, b and c; LUNDBORG 1963) and found to be effectively inhibited by reserpine, it is most probable that there exists another mechanism, distinct from the former, which is not inhibited by reserpine and which is sensitive to cocaine. The latter mechanism—the membrane mechanism—functions in the entire adrenergic neuron, since the monoamines taken up and accumulated by this mechanism are found uniformly distributed in all parts of the neuron. Its subcellular localization has not definitely been shown, but since it is located over the entire neuron and since its inhibition by cocaine seems to imply that the entrance of monoamines into the adrenergic nerves is prevented, it is most conceivable that it is located in or at the level of the cell membrane.

The topographical localization of the two uptake-concentration mechanisms indicates that the amine granules take up monoamines located intraneuronally, while the membrane mechanism takes up extraneuronally located monoamines.

In the present investigation, the increase in the NA content of the adrenergic nerves following the administration of exogenous NA was very short lasting. This indicates that the granules have a limited storage capacity and that an excess of NA is metabolized by MAO, since pretreatment with nialamide prolongs the increase. This is in agreement with other investigations (MUSCHOLL 1960) and it also has been found with the use of labelled NA that the exogenous NA mixes with endogenous NA and stays in the granules for a relatively long time (MONTANARI, COSTA, BEAVEN and BRODIE 1963; AXELROD 1964).

Several authors have proposed another uptake mechanism in the adrenergic nerves, distinct from that of the amine granules (CARLSSON, HILLARP and WALDECK 1963), but its nature, properties and localization have remained obscure owing to the indirect methods used to study it, and it has been impossible to assign with certainty the properties of the intact adrenergic nerves to either of the two mechanisms. With the fluorescence method it is possible to obtain more detailed information on the extragranular mechanism, the membrane mechanism, if interference from the uptake mechanism of the amine is reduced to a minimum by pretreatment of the animals with reserpine, which has no or very little effect on uptake by the membrane mechanism.

As already pointed out, the membrane mechanism is very rapid and efficient in concentrating CA against a very high concentration gradient. NA (α-methyl-NA) seems to be more readily taken up than DA, while 5-HT is very poorly taken up by the membrane mechanism, contrary to

the case in the amine granules (BERTLER, ROSENGREN and ROSENGREN 1960). On the other hand, the difference in uptake as between the stereoisomers which was found in the amine granules (CARLSSON, HILLARP and WALDECK 1963; EULER, STJÄRNE and LISHAJKO 1963; EULER and LISHAJKO 1964) does not seem to exist in the action of the membrane mechanism (cf. CROUT 1962; KOPIN and BRIDGERS 1963; NASH, COSTA and BRODIE 1963; BEAVEN and MAICKEL 1964).

As moreover the extragranular uptake can be prevented by a number of different agents, it seems completely clear that the uptake by the membrane mechanism is an active process and not a simple passive diffusion process. The same conclusions have recently been obtained from *in vitro* studies on slices of brain, vas deferens and submaxillary gland (HAMBERGER and MASUOKA 1965).

It seems obvious from the present investigation that the main function of the membrane mechanism is normally to recapture the transmitter released from the adrenergic nerves. The recapture is clearly very important for the transmitter economy. The membrane mechanism probably also subserves the rapid inactivation of extraneuronal NA—circulating or in close contact with the adrenergic receptor—which several authors (see TRENDELENBURG 1963 *b*) have suggested to be achieved by tissue binding rather than direct enzymatic metabolism. This latter view is consequently supported by the findings in the present paper.

As increased nerve activity causes no or only a slight reduction in the amount of NA (or α-methyl-NA) taken up in the adrenergic nerves in reserpinized animals, it seems obvious that either there is no increase or qualitative change in the release of the extragranularly located NA (or α-methyl-NA) on nerve impulses, or else the capacity of the membrane mechanism is great enough to recapture the increased release of NA (α-methyl-NA). There is, however, other evidence which indicates that binding to the granules is necessary for an adequate release of the transmitter (see CARLSSON 1964) and that extragranularly located amines are not readily available for release on nerve impulses.

Even if there is no accumulation of monoamines in a denervated iris where the axons have degenerated, it cannot be excluded that the effector cells or the cells of Schwann which cover most of the surface of the adrenergic nerves have some importance for the uptake of exogenous CA. It is likewise possible that the release of NA (or α-methyl-NA) is influenced by the cells of Schwann and that these are important for the further fate of the released transmitter. It is remarkable that the adrenergic axon at the varicosities which are supposed to be the sites of the release of the trans-

mitter on nerve impulses is partially not covered by the Schwann's sheath being naked towards the extracellular space the synaptic cleft between the nerve and the effector cell (NILSSON 1964)

MAO

For an adequate evaluation of the significance of the action of MAO normally and under different experimental conditions, it is necessary to know the exact localization—intraneuronal *versus* extraneuronal—of the enzyme responsible for the deamination which latter is an important step in the metabolism of the intraneuronal monoamines

Earlier investigations with the fluorescence method (HAMBERGER MALMFORS NORBERG and SACHS 1964 HILLARP and MALMFORS 1964 NORBERG *1965a) strongly indicated an intraneuronal localization,* which was in agreement with certain other assumptions made after investigations using different approaches (CARLSSON POSENGREN BERTLER and NILSSON 1957 CARLSSON 1960 SPECTOR KUNTZMAN SHORE and BRODIE 1960 CARLSSON and HILLARP 1962) The present investigation however produced certain findings which give more definite support for an intraneuronal localization

a) DA (1 mg/kg i v) given to reserpinized animals pretreated with malamide caused a moderate to strong fluorescence in the adrenergic nerves (see chapter V) If the animals were not pretreated with malamide (see chapter V) or if they were pretreated also with desmethylimipramine (see chapter IX) there was no specific fluorescence at all in the former case presumably due to the destruction of DA by MAO and in the latter to prevention of the DA uptake by desmethylimipramine If DA was replaced by L DOPA in these experiments (see chapter V) the same results were obtained except that desmethylimipramine was unable to prevent restitution of the specific fluorescence

These results strongly indicate an intraneuronal localization of MAO Otherwise the lack of specific fluorescence after the administration of L DOPA alone to reserpinized animals cannot be due only to a very rapid release of the formed DA (see chapter V) since inhibition of its reuptake (by pretreatment with desmethylimipramine)—which would be very effective and responsible for the restituted fluorescence after DA preceded by malamide if MAO is suggested to have an extraneuronal localization—does not markedly reduce the intensity of the restituted fluorescence after L DOPA preceded by malamide (see chapter V)

b) If MAO is effectively inhibited by a short lasting MAOI e g the reversibly acting harmine (UNDENFRIEND WITKOP REDFIELD and WEISS

BACH 1958) the accumulated NA in reserpinized animals disappears more rapidly than when the long lasting irreversible malamide is used, even in combination with an inhibition of the recapture of the membrane mechanism. If the recapture of the released NA is complete or nearly complete—which however is uncertain—this argues for an intraaxonal metabolism of the accumulated NA by MAO when the MAO inhibiting effect of harmine is decreased and consequently for an intraaxonal localization of the MAO.

c) *When reserpine was preceded by malamide the depletion of NA was delayed or even diminished*, which agrees with other findings that metabolism by MAO is the main breakdown pathway of the endogenous NA in depletion by reserpine (CARLSSON 1960, SPECTOR, KUNTZMAN, SHORE and BRODIE 1960, ANDÉN, ROOS and WERDINIUS 1964). Since reserpine administration alone produces no or under special circumstances only very weak sympathomimetic effects, it is obvious that the NA must be metabolized by intraneuronally located MAO or—less probably—by extraneuronally located MAO immediately outside the axon membrane.

From the experiments above described and from other experiments performed with the fluorescence method, it seems reasonable to assume that MAO really is located intraneuronally, a view that is further supported by e.g. the decrease of the MAO activity in adrenergically innervated tissues after denervation (BURN and ROBINSON 1952), the histochemical demonstration of MAO activity in at least the cell bodies of the sympathetic ganglion cells (KOELLE and VALK 1954, NORBERG unpublished observations) and the absence of any potentiated or prolonged effect of nerve stimulation after MAO inhibition.

Even if all data speak in favour of an intraneuronal localization of the MAO, it is possible that there is MAO also just outside the adrenergic nerves and that this is partly responsible for the extraneuronal enzymatic elimination of NA. *However, the extraneuronal CA could also be broken down by the intraneuronal MAO after having been taken up by the membrane mechanism of the adrenergic nerves.*

Reserpine

All effects of reserpine on the adrenergic nerves are still uncertain and there will be discussed here only its effects on the amine granules and on the membrane mechanism on the basis of the findings made in the present study.

It has earlier been convincingly shown that reserpine effectively inhibits

CONCLUSIONS AND SUMMARY

In the present investigation the fluorescence method of FALCK and HILLARP as applied to the adrenergic nerves in the rat iris prepared as a whole mount has proved to be an extremely useful tool for the study of the morphology physiology and pharmacology of the adrenergic nerves In some cases it has confirmed earlier findings in others added important information thanks to its particular direct nature and in still other cases it has furnished new and fundamental knowledge on the adrenergic nerves The most essential conclusions are given below

1 For the first time it has been possible to visualize the distribution of an individual adrenergic neuron in an effector organ and to obtain direct morphological evidence for the supposed functional organization of the autonomic innervation apparatus

Conclusive evidence has been obtained that NA the adrenergic transmitter is released from the adrenergic nerves upon nerve impulses and that the varicosities which store most of the NA present in the adrenergic nerves are probably the sites for this release within the entire final varicose branches of the adrenergic axon These are consequently the true adrenergic terminals on both a functional and morphological basis Systems of branching terminals which form a morphological and functional unit are joined together in a net work the adrenergic ground plexus (HILLARP 1946 1959) which is considered to be the true innervation apparatus (see also NORBERG and HAMBERGER 1964)

2 In the adrenergic nerves of normal animals most of the NA is probably located to the amine granules with very little situated extragranularly in the cytoplasm as the MAO in all probability shown to be present intra axonally very effectively and rapidly metabolizes the transmitter unless the latter is protected in the amine granules from MAO On the other hand the NA that accumulates in the adrenergic nerves in reserpinized animals after treatment with a potent MAOI is in all probability located extragranularly in the cytoplasm either loosely attached to certain structures or just free in the cytoplasm since reserpine is known to effectively prevent the uptake of NA into the amine granules

3 It has been definitely shown that exogenous NA can be taken up by

the adrenergic nerves and that there are at least two different uptake concentration mechanisms for CA in the adrenergic nerves. One is situated in the amine granules and inhibited very effectively by reserpine, while the other is located in all probability in the cell membrane over the whole neuron and is blocked by e.g. cocaine, desmethylimipramine, imipramine, chlorpromazine, guanethidine, tyramine and metaraminol but apparently not affected by reserpine, ouabain or phenoxybenzamine.

4. As the membrane mechanism has been found to be very rapid and effective in taking up NA, it seems reasonable to assume that it is of great importance for the transmitter economy and the inactivation of extraneuronal NA at the adrenergic receptors. This is further supported by the fact that its inhibition or disappearance—the latter on the degeneration of the adrenergic nerves simultaneously with or shortly before the disappearance of their endogenous NA—potentiates the effect of exogenous NA and that its inhibition after the accumulation of extragranular NA in the cytoplasm accelerates the spontaneous disappearance of the NA.

5. With the help of inhibitors of the NA biosynthesis it has been established that the synthesis is important for the maintenance of a constant transmitter level when the nerve activity is relatively high. The rate of depletion by reserpine is increased at increased nerve activity, presumably due to an increased turnover of NA in the amine granules.

The amine granules seem furthermore to be important for the release of the transmitter on nerve impulses, since no or a very little decrease in the extragranularly located NA is found in reserpinized animals even after intense nerve stimulation.

From these conclusions the following summary may be made.

The sympathetic adrenergic nerves can synthesize, take up, store, release and metabolize NA, which serves as their transmitter substance. The nerves come into contact with the effectors by a ground plexus composed of the final branches, the terminals, where the varicosities are the specialized structures for the synthesis, storage and release of transmitter.

In the varicosities there are accumulated granules which take part in the synthesis, store the main part of the transmitter in very high concentrations and probably take direct part in the release of the transmitter at nerve impulses. Extragranularly in the cytoplasm there is present MAO which effectively metabolizes the NA that is either released from the granules into the cytoplasm or taken up by a specialized mechanism in the axon membrane. This mechanism is important in maintaining a constant level of the transmitter in the nerves and inactivates extraneuronal NA by taking it up into the nerves.

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FIGURES

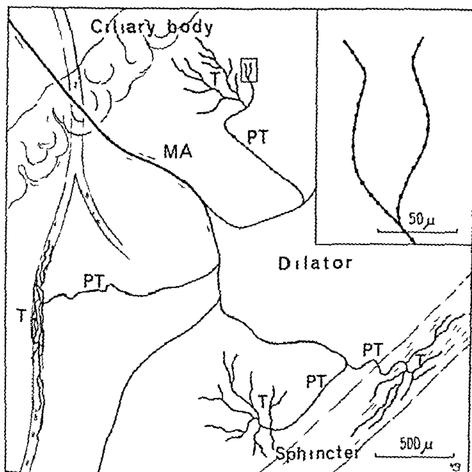


FIG 1 A schemat pi ture of how th axon of an ndiv i lual adrenergic neuron extends throughout the ir The drawin n ade uj of pieces from d fferent preparation In th upper l ft ha l corner there s seen apart from an arteriole a single main axon (MA) wh ch pa th ciliary bod fr m th l oro I ı a small nerve bun lle It then branche int thin fil res ext n l ng ove a large part of the iris (cf Fig 4) These thin branches th preterminal ax ns (PT) late change tl eir appearance showing small irregular e l ments The pr terminal axon f nally spl t up into a system of branch ing fibres (Fig 3) the term nals (T) These have a characteri tic appearance with abu t more or less oval enlargements or var o iti s (Cf the enlarged drawing of branch terminals in the upper r ght hand corn Systems of branching termi nals fron ne an l the same main axon can be fou d in widely different parts of the dilator le in the sphincter region and arou l an arteriole

FIG 2 Pat ms 45 h after a partial d n rvation A system of branching terminal ari ing from a preterminal axon i seen over the dilator × 160

FIG 3 Pat rn 45 h aft r a partial d r a t A sy t m f branching t rminals ari ing from a pret rmi al ax n ( ) i seen v r th dilat r 160

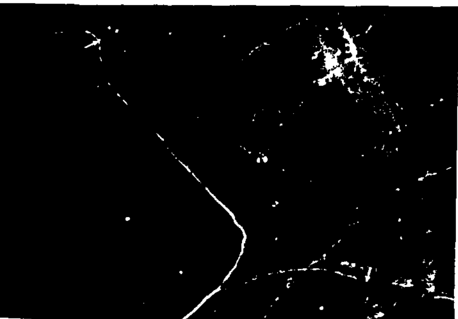


Fig 4 Rat iris 48 h after a partial d n rvation A main axon is seen branching into preterminal axons which also branch (→) × 160



Fig 5 I at iris 48 h after a partial d nervation At least two sy tems of branching terminals are seen together with numerous preterminal axons 160

Fig 6 Iris of normal rat In addition to the strongly fluorescent and uniformly distributed plexus of adrenergic nerve terminals over the dilator muscle there is seen an arteriole surrounded by a similar adrenergic ground plexus ×160

Fig 7 Iris of normal rat To the left is seen the adrenergic ground plexus of the dilator (D) intimately connected with the plexus of terminals over the sphincter (S) Notice the different main direction of the terminals over the muscle ×160

FIG 8 Iris of normal rat The strands of the ground plexus over the dilator consist of one or several terminals with pronounced varicosities Some weakly fluorescent non terminal axons are seen (→) × 250

FIG 9 Iris of guinea pig Over the dilator is seen the plexus of ...

Fig. 10. Iris of normal rat. This is the appearance of the terminals under normal optimum conditions for the fluorescence method—the formaldehyde reaction was performed with paraformaldehyde equilibrated at 70 per cent relative humidity. The varicosities are distinct and have a very high intensity. ×400

Fig. 11. Iris of normal rat. The formaldehyde reaction was performed with paraformaldehyde equilibrated at 10 per cent relative humidity. The varicosities are distinct and have a high intensity while the segments between them are in some places hardly seen. Approximately the same exposure as in Fig. 10. ×400

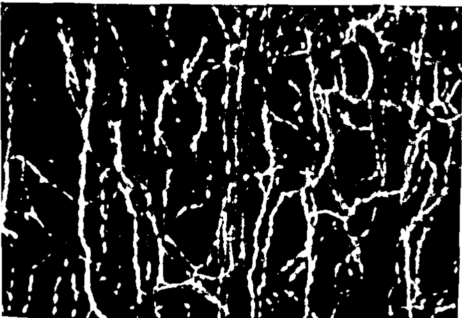


Fig 1 Iris of normal rat The formaldehyde reaction was performed with parafor maldehyde equilibrated at 90 per cent relative humidity The transmitter and/or the fluorescent product have diffused giving the varicosities and terminals a broad and diffuse appearance × 400

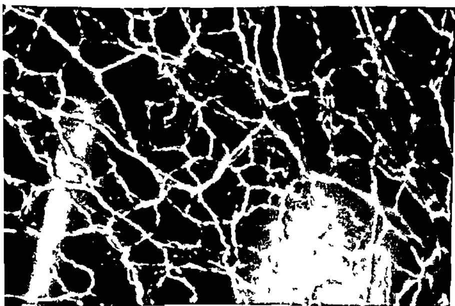


Fig 13 Iris of normal rat The formaldehyde reaction was performed with paraformal dehyde equilibrated at 90 per cent relative humidity The diffusion of the transmitter and/or the fluorescent product seems to be limited to within the sheath of Schwann × 400

Fig 14 Rat iris Animal treated with reserpine (10 mg/kg i p) 1 h before death The fluorescence intensity of the terminals is somewhat lower than normal but the varicosities are still very pronounced Slightly longer exposure than in Fig 8 × 250

Fig 15 Rat iris Animal treated with reserpine (10 mg/kg i p) [illegible] h before death The fluorescence intensity is low but the varicosities are still clearly seen Longer exposure than in Fig 14 × 250

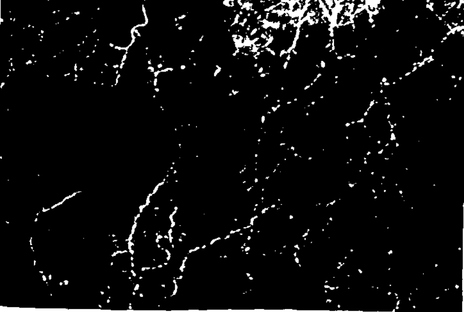


Fig 16 Rat iris Animal treated with reserpine (10 mg kg i p) 3 h before death Some weakly fluorescent continuous fibres remain while the fluorescence of most of the terminals has disappeared Longer exposure than in Fig 14 × 250

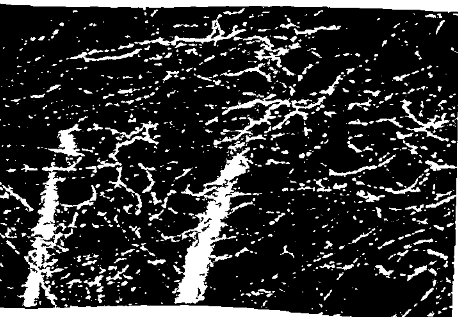


Fig 17 Rat iris Animal treated with reserpine (10 mg/kg i p) 7- h before death The fluorescence intensity has returned to almost normal level and the varicosities show the same appearance as during depletion. Approximately the same exposure as in Fig 14 × 250



Fig. 18 Iris of normal rat. The uniform adrenergic ground plexus is seen over the dilator. The terminals have pronounced varicosities. A bundle of weakly fluorescent non-terminal axons is also present (→). ×160

Fig. 19 Rat iris. Animal treated with reserpine (mg/kg, i.p.) 18 h, nialamide (100 mg/kg, i.p.) 3 h and NA (0. mg/kg, i.v.) 1 min before death. The fluorescent fibres form a plexus of the same appearance as in Fig. 18 except that the pronounced varicosities are missing. The strikingly fluorescent fibres running in some places in bundles correspond to the weakly fluorescent non-terminal axons in untreated animal. Approximately the same exposure as in Fig. 18. ×160

Fig. 20 Rat iris Animal treated with reserpine (5 mg/kg i p) 18 h nialamide (100 mg/kg i p) 3 h and DA (10 mg/kg i p) 1 h before death The fluorescent fibres form a plexus of the same appearance as in Fig 18 The varicosities are somewhat less pronounced than in Fig 18 but more so than in Fig 19 Some strikingly fluorescent non terminal axons are seen (→) Approximately the same exposure as in Fig 18 ×160

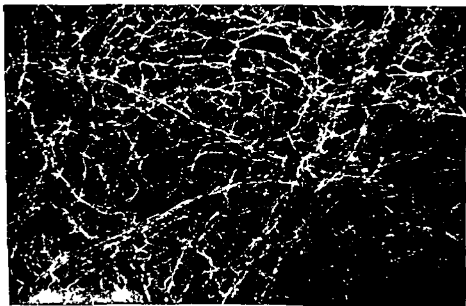


Fig. 21 Rat iris Animal treated with nialamide (100 mg/kg i p) 5 h before death The terminals in the ground plexus show an increased intensity and pronounced varicosities The non terminal axons (→) are more evident than in the iris of normal animals Approximately the same exposure as in Fig 18 ×160



FIG. 22. Iris of normal rat. One or several terminals with intensely fluorescent varicosities are seen forming the strands of the adrenergic ground plexus. Some weakly fluorescent non-terminal axons are also seen (→). × 400.

FIG. 23. Rat iris. Animal treated with nialamide (100 mg/kg i.p.) and 3 h and NA (0.2 mg/kg i.v.) [illegible] min before death. The segments between the varicosities in the terminals have a stronger fluorescence than in Fig. 22, giving the terminals a somewhat smoother appearance. The non-terminal axons (→) have also a higher intensity. Approximately the same exposure as in Fig. 22. × 400.

Fig 4 Rat iris Animal treated with reserpine (5 mg/kg i p) 18 h nialamide (100 mg/kg i p) 3 h and DA (–0 mg/kg i p) 1 h before death The fluorescent fibres in the plexus—the terminals—have varicosities that are less pronounced than in Fig – but more so than in Fig –5 The non terminal axons (→) are easily seen Approximately the same exposure as in Fig –– × 400

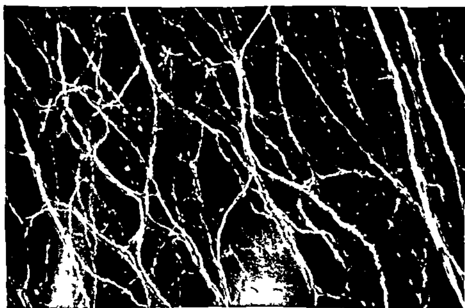


Fig 25 Rat iris Animal treated with reserpine (5 mg/kg i p) 18 h nialamide (100 mg/kg i p) 3 h and NA (0 1 mg/kg) 15 min before death The fluorescent fibres in the plexus—the terminals—have no or rather light varicosities The non terminal axons (→) are easily seen Approximately the same exposure as in Fig –– × 400

Fig 22 Iris of normal rat One or several terminals with intensely fluorescent varicosities are seen forming the strands of the adrenergic ground plexus Some weakly fluorescent non terminal axons are also seen (→) × 400

Fig 23 Rat iris Animal treated with nialamide (100 mg/kg i p) and 3 h and NA (0 2 mg/kg i v) 15 min before death The segments between the varicosities in the terminals have a stronger fluorescence than in Fig 22 giving the terminals a somewhat smoother appearance The non terminal axons (→) have also a higher intensity Approximately the same exposure as in Fig 22 × 400

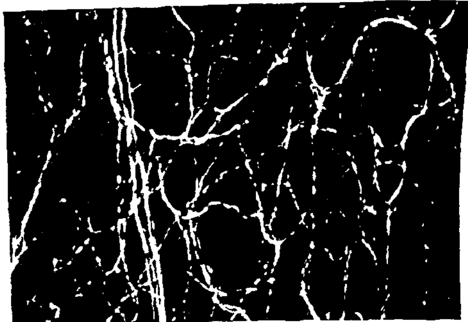


Fig 4 Rat iris Animal treated with reserpine (5 mg/kg i p) 18 h nialamide (10 mg/kg i p) 3 h and DA (0 mg kg i p) 1 h before death The fluorescent fibres in the pl xus—the terminal —have varicosities that are less pronounced than in F 22, but more so than in Fig 5 The non terminal axons (–) are easily seen Approximately the same exposure a in Fi – 400

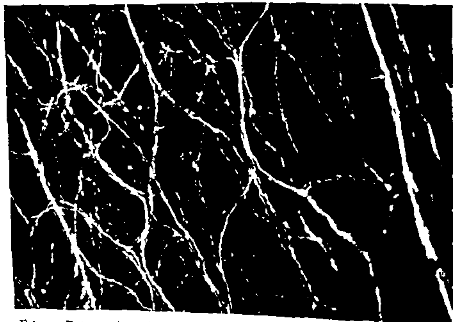


Fig Rat iris Animal treated with reserpine (mg/kg i p) [illegible] mg/kg i p) 3 h and NA (0 1 mg/kg i v) 15 min before death [illegible] in the pl xus—the terminals—have no or rath r slight [illegible] axons (→) are easily seen [illegible]

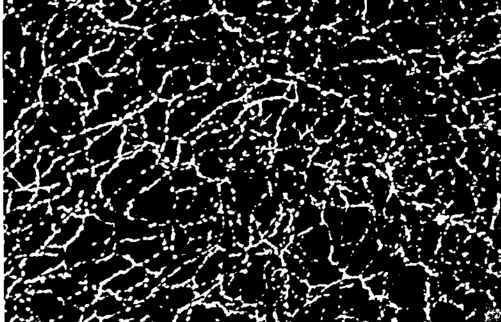


FIG. 30 Normal mouse iris The strands of the adrenergic groundplexus over the dilator consist for the most part of one terminal only having large and intensely fluorescent varicosities The segments between the varicosities like the non terminal axons (→) have a weak to very weak fluorescence × 250

FIG. 31 Mouse iris Animal treated with nialamide (100 mg/kg i.p.) 3 h and NA (5 mg/kg i.p.) 15 min before death The adrenergic nerves show an increased fluorescence in comparison with Fig 30 Particularly the fluorescence of the segments between the varicosities has increased giving the terminals a somewhat smoother appearance Even the non terminal axons (→) however have a higher intensity making them

Fig 32 Mouse iris Animal treated with reserpine (10 mg/kg i p) 18 h nialamide (100 mg/kg i p) 3 h and DA (10 mg/kg i p) 1 h before death The plexus of the fluorescent fibres shows the same arrangement as in Fig 30 while the fibres—the terminals—have varicosities which are not as pronounced as in Fig 30 although more so than in Fig 33 The broad and strongly fluorescent fibres (→) are clearly non terminal axons Approximately the same exposure as in Fig 30 ×250

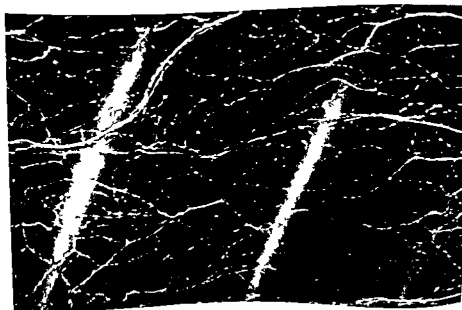


Fig 33 Mouse iris Animal treated with reserpine (10 mg/kg i p) 18 h nialamide (100 mg/kg i p) 3 h and NA (5 mg/kg i p) 15 min before death The fluorescent fibres have the same arrangement as in Fig 30 but the varicosities of the fibres—th

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CONTENTS

The present thesis is based on the following publications

I ELMQVIST D T R JOHNS and S THESLEFF, A study of some electro physiological properties of human intercostal muscle *J Physiol* (Lond) 1960 *154* 602–607

II DAHLBACK, O D ELMQVIST T R JOHNS S RADNER and S THESLEFF, An electrophysiological study of the neuromuscular junction in myasthenia gravis *J Physiol* (Lond) 1961 156 336–343

III ELMQVIST D and D M J QUASTEL Presynaptic action of hemicholinium at the neuromuscular junction *J Physiol* (Lond) 1965 In press

IV ELMQVIST D W W HOFMANN J KUGELBERG and D M J QUASTEL An electrophysiological investigation of neuromuscular transmission in myasthenia gravis *J Physiol* (Lond) 1964 *174* 417–434

V ELMQVIST D and D M J QUASTEL A quantitative study of end plate potentials in isolated human muscle *J Physiol* (Lond) 1965 In press

VI ELMQVIST D Potassium induced release of transmitter at the human neuro muscular junction *Acta physiol scand* 1965 In press

The above papers will be referred to as Paper I–VI

INTRODUCTION

THE DEVELOPMENT OF THE PRESENT CONCEPT OF NEURO MUSCULAR TRANSMISSION IN HEALTH AND DISEASE

Normal

As the resolving power of the microscope was improved at the beginning of the nineteenth century the relationship between nerve and muscle was investigated DOYERE (1840) was the first to propose the now well established fact that the nerve fibre comes to a complete termination on the surface of the muscle fibre It was implicit in this theory that the nerve terminals must functionally connect with the muscle fibres by contact rather than by continuity

DU BOIS REYMOND in 1877 discussed the possibilities that transmission of impulses from nerve to muscle at the neuromuscular junction or end plate could be either electrical or chemical On reading his work one gets the impression *that he favoured the chemical hypothesis* KUHNE (1888) *on the other hand* was of the opinion that the action current in the motor nerve excited the motor end plate electrically

The theory of chemical transmission across synapses was made more substantial in 1904 when ELLIOT suggested that smooth muscle fibres were excited by adrenaline liberated from the sympathetic nerve terminal Furthermore DIXON in 1906 suggested that a substance which had an action similar to muscarine was released from the peripheral parasympathetic neurones Experimental evidence for the existence of a chemical transmitter was provided by LOEWI (1921) who showed that a substance which mimicked vagus stimulation was liberated when the vagus nerve was stimulated This substance was identified as acetylcholine In 1936 DALE FELDBERG and VOGT made it probable that acetylcholine also was the transmitter at neuromuscular junctions

The electrical events associated with synaptic transmission at the neuromuscular junction were first demonstrated by GOPFERT and SCHAEFER (1938) and SCHAEFER and HAASS (1939) They showed that upon stimulation of the motor nerve a surface negative potential was elicited at the end plate region (Endplattenstrom end plate potential) which if great enough triggered an action potential in the muscle fibres The amplitude of this potential was reduced by increasing the frequency of nerve stimulation and by treating the preparation with curare They failed to realise that this potential was caused by release of acetylcholine from the motor nerve terminals but interpreted their results as indicating an electrical transmission between the motor nerve terminals and the muscle end plates

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As is now generally accepted (ECCLES 1964) the word synapse and its derivative forms have been applied here to the neuromuscular junction

In their studies of the effects of curare and eserine on the end plate potenti[al] ECCLES KATZ and KUFFLER (1941 1942) concluded that the end plate potential was caused by acetylcholine liberated from the motor nerve terminals rath[er] than electrically by the action potential in the nerve

The introduction of glass capillary microelectrodes filled with an electroly[te] (usually KCl) (GRAHAM and GERARD 1946 LING and GERARD 1949) provide[d] the temporal and spatial discrimination required for studies of the mechanism of the secretory process by which acetylcholine is released from the motor nerve terminals and how it acts upon the end plate of the muscle fibre The application of techniques of intracellular recording and of methods for iontophoret[ic] microapplication of drugs (NASTUK 1953) greatly expanded our knowledge [of] both the pre and post synaptic events responsible for the transmisson of impuls[es] from the motor nerve to the muscle fibre

In single muscle fibres BUCHTHAL and LINDHARD (1937) and KUFFLER (194[?]) demonstrated that the stimulating action of acetylcholine was restricted to th[e] synaptic area Very localized application of acetylcholine from micropipett[es] (NASTUK 1951 1953 1954) confirmed these results Intracellular applicatio[n] of acetylcholine even at the end plate region did not induce any depolariza[tion] of the muscle fibre (DEL CASTILLO and KATZ 1955) It was concluded th[at] the receptors for acetylcholine are situated only on the surface of the muscl[e] fibre in the end plate region Upon chronic denervation the muscle fibre becam[e] sensitive to acetylcholine all over its surface (AXELSSON and THESLEFF 195[9] MILEDI 1960)

End plate potentials and potentials caused by iontophoretic microapplicatio[n] of acetylcholine were affected similarly by curare and cholinesterase inhibitor[s] The latter enzyme which hydrolyzes acetylcholine has been localized at th[e] motor end plate by KOELLE and FRIEDENWALD (1949) among others Th[e] cholinesterase inhibitors enhanced and strikingly prolonged the duration [of] the potentials while curare decreased their amplitude without altering the[ir] time course The effect of curare was presumably due to a competition betwee[n] this drug and acetylcholine for acetylcholine receptors at the motor end plat[e] (DEL CASTILLO and KATZ 1957 JENKINSON 1960)

Intracellular recording at the unstimulated neuromuscular junction reveale[d] small spontaneous depolarizations with a fairly uniform amplitude miniatu[re] end plate potentials which were confined to the end plate region (FATT an[d] KATZ 1952 BOYD and MARTIN 1956a LILEY 1956a) The miniature end plat[e] potentials represented the postsynaptic response to acetylcholine spontaneousl[y] liberated from the motor nerve terminals as seen from their identities in tim[e] course and in the effects of pharmacological agents such as curare and eserin[e] to the behaviour of end plate potentials (FATT and KATZ 1952 DEL CASTILL[O] and KATZ 1956 LILEY 1956a) DEL CASTILLO and KATZ (1956) conclude[d] that these miniature potentials represented the smallest unit of spontaneou[s] release of acetylcholine These units contained many molecules of acetylcholin[e] since the application of low concentrations of acetylcholine gave continuousl[y]

graded depolarizations and the amplitude of the miniature end plate potentials gradually decreased with increasing concentration of curare Neither of these manipulations affected their frequency as would be expected if each miniature end plate potential was caused by a single acetylcholine molecule

The frequency of the miniature end plate potentials at most unstimulated neuromuscular junctions about 1/sec was greatly increased when the motor nerve terminals were depolarized either by electric current (DEL CASTILLO and KATZ 1954 c LILEY 1956 c) or by increasing the external potassium ion concentration (LILEY 1956 c FURUKAWA FURUKAWA and TAKAGI 1957 TAKEUCHI and TAKEUCHI 1961) LILEY (1956 c) found that a 2 7 fold increase in external potassium ion concentration (above 10 mM) resulted in a 55 fold increase in the frequency of the miniature end plate potentials KATZ (1962) recalculated LILEY s measurements and found that there was a linear relationship between the calculated membrane depolarization and the logarithm of the frequency of the miniature end plate potentials

An action potential in the motor nerve also induces the release of acetyl choline from the terminals in the form of quanta of the same size as those responsible for the miniature end plate potentials A single action potential in the motor nerve has been estimated to release hundred or more such quanta (DEL CASTILLO and KATZ 1954 a MARTIN 1955 BOYD and MARTIN 1956 b LILEY 1956 b) If the linear relation between membrane depolarization and logarithm of the frequency of the miniature end plate potentials holds for the very short and great depolarization that occurs during the action potential in the motor nerve terminal it could account for release of the great number of acetylcholine quanta normally occurring as the result of a nerve stimulus (LILEY 1956 c)

During repetitive indirect stimulation of mammalian neuromuscular junctions the amplitude of the end plate potentials declined during the first few impulses of a tetanus They were then maintained at a fairly constant level which was inversely dependent upon the frequency of stimulation (LUNDBERG and QUILISCH 1953 a LILEY and NORTH 1953) LILEY and NORTH (1953) attributed the decline in the amplitude of the end plate potentials seen in the beginning of a tetanus to depletion of the nerve terminal of acetylcholine available or free for immediate release The constant level then obtained represented the rate at which acetyl choline was made available for liberation THESLEFF (1959) suggested that desensitization of the receptors at the postjunctional membrane might con tribute to the fall in the amplitude of the end plate potentials during repetitive nerve stimulation When the number of acetylcholine quanta liberated by a nerve impulse was greatly reduced by excess magnesium and/or deficient cal cium in the external medium no depletion of the amounts of acetylcholine avail able for release was expected In such experiments the amplitude of the end plate potentials increased in the beginning of the tetanus (LUNDBERG and QUILISCH 1953 b DEL CASTILLO and KATZ 1954 b LILEY 1956 b HUBBARD 1963) Increased amplitude of the end plate potentials was found for a period

after the end of the tetanus (post tetanic potentiation) During the stimulation and the period of post tetanic potentiation spontaneous quantum release recorded as miniature end plate potentials was increased to the same degree (LILEY 1956 a BROOKS 1956 HUBBARD 1959 1963) HUBBARD and SCHMIDT (1962) showed that the action potential in the motor nerve terminals was increased during this period The facilitation by stimulation was not dependent on transmitter release but was increased with greater frequency and duration of the stimulation (DEL CASTILLO and KATZ 1954 b DUDEL and KUFFLER 1961 HUBBARD 1963) The amount of acetylcholine released by a nerve stimulus during and after a tetanus is a function of facilitation of release and depletion of available acetylcholine

The finding that hyperpolarization of the motor nerve terminals occurred during repetitive nerve stimulation (HUBBARD and SCHMIDT 1963) and that an increase in transmitter mobilization could be induced by artificial hyperpolarization (HUBBARD and WILLIS 1962) made ECCLES (1964) suggest that an increase in transmitter mobilization is of great importance for the facilitation of acetyl choline release during and after repetitive nerve stimulation LILEY and NORTH (1953) observed that the rate of decline of the amplitude of successive end plate potentials in a tetanus was more rapid when the tetanus was preceded by a conditioning tetanus They inferred that this finding showed that during post tetanic potentiation an increased fraction of the acetylcholine free or available for release was liberated by a nerve impulse LUNDBERG and QUILISCH (1953 b) observed a similar increase in the rate of decline in the amplitude of successive end plate potentials when the external calcium concentration was increased

It was concluded that the basic unit of transmitter release is the acetylcholine quantum The amount of acetylcholine in a quantum has hitherto been found constant and not affected by experimental procedures The amount of transmitter release from the motor nerve terminals has thus been thought to be dependent only on the number of quanta extruded KATZ (1962) has stated "the frequency of the miniature potentials is controlled entirely by the conditions of the presynaptic membrane while their amplitude is controlled by the properties of the postsynaptic membrane"

The amount of acetylcholine liberated by a nerve impulse appeared to be regulated by at least three factors i) the amount of transmitter in the nerve terminal free or available for release ii) the fraction of this amount which is liberated by a nerve impulse and iii) the extent to which mobilization is able to keep up with release

Myasthenia Gravis

Myasthenia gravis is a chronic disease characterized by muscular weakness of fluctuating intensity It is aggravated by activity and improved by rest The weakness is not associated with any significant atrophy of the muscles at least in the earlier stages of disease

The disease was distinguished as a clinical entity mainly by ERB (1879) and by GOLDFLAM (1893) whose clinical characterization of the disorder is to great extent still valid

The symptoms of myasthenia gravis are primarily due to dysfunction of the motor system The muscles of the eyes the larynx and of mastication are often affected first and most seriously Later the muscles of the trunk and extremities may become involved less often the symptoms of myasthenia gravis first appear in these muscles Characteristically an involved muscle rapidly becomes progressively weaker upon exercise Most patients are better in the morning than in the afternoon Remissions and relapses occur Paralysis of the respiratory muscles may cause the death of patients with myasthenia gravis

In 1934 MARY WALKER noted the similarity between the symptoms shown by laboratory animals treated with curare and those of patients suffering from myasthenia gravis She therefore treated a myasthenic patient with eserine with a therapeutic effect Since WALKER's observation it has generally been accepted that the muscular weakness is caused by a neuromuscular blockade Soon after the introduction of tubocurarine as a muscle relaxing agent in anesthesiology it was found that myasthenic patients were extremely sensitive to the drug and in 1943 BENNET and CASH introduced curare as a diagnostic test for myasthenia gravis The supersensitivity of myasthenic muscle to tubocurarine has been confirmed *in vitro* by DILLON HERRMANN BARKER and SABAWALA (1961)

The three most widely discussed theories for the mechanism underlying the neuromuscular block in myasthenia gravis are i) the amount of acetylcholine synthesized or released by the motor nerve terminals is less than normal ii) the acetylcholinesterase activity at the neuromuscular junction is abnormally active preventing the acetylcholine liberated from the motor nerve terminals from reaching the acetylcholine receptors at the postjunctional membrane in an effective concentration iii) the postjunctional membrane is less sensitive to acetylcholine either because of a defect of the receptors or because of the presence of an inhibiting (curare like) substance

TORDA and WOLFF (1943 1945) first suggested that an impaired synthesis of acetylcholine in the motor nerve terminals might occur in myasthenic patients on the basis of their observation that serum from patients produced an inhibition of choline acetylase *in vitro* Other experiments leading to similar conclusions were those of DESMEDT (1957 1958) He tested the degree of neuromuscular blockade in myasthenic patients after a conditioning tetanus with infrequent nerve stimulation and recorded the resulting electromyogram or muscle contraction After a period of decrease in the neuromuscular blockade there was a delayed increase in the block He compared these results with those obtained from similar experiments in the cat and found that in curarized animals no such phenomena occurred In cats treated with hemicholinium (HC-3) a drug known to block acetylcholine synthesis in intact nervous tissue a delayed increase in the neuromuscular blockade was produced DESMEDT therefore suggested that in myasthenia gravis there might be an impaired acetylcholine synthesis According

to him this could be brought about by a circulating substance similar to HC-3 or by an inadequate supply of the enzyme choline acetylase There is as yet no information about the actual amount of acetylcholine liberated from the myasthenic motor nerve endings during stimulation

The therapeutic efficacy of cholinesterase inhibiting drugs (WALKER 1934) could support the hypothesis that acetylcholine esterase activity at myasthenic neuromuscular junctions was increased No experimental basis for this hypothesis has been found Histochemical studies of cholinesterase have shown similar distribution and activity at normal and myasthenic end plates (COERS 1961 BLUMBERG ZACKS and BAUER 1961 cf FOLDES and MCNALL 1962) No ab normality in the cholinesterase activity of myasthenic serum or muscle has been found (WILSON MAW and GEOGHEGAN 1951)

In 1938 MARY WALKER reported that in myasthenia gravis a substance blocking neuromuscular transmission was produced when the muscles were activated On exercising the arm muscles after interruption of the venous return with a tourniquet release of the cuff induced ptosis and generalized weakness Some authors have been unable to reproduce these results while others have confirmed them in a few patients (OSSERMAN 1958 WILSON and STONER 1944 BERGH 1953) The occurrence of transient neonatal myasthenia in newborn children of myasthenic mothers has been taken as additional evidence for the presence of a circulating neuromuscular blocking substance in myasthenic patients

These sparse but striking observations have led many investigators to search for neuromuscular blocking substances in blood muscle and thymus from patients with myasthenia gravis (critically reviewed by BERGH 1953) The studies were performed using nerve muscle preparations from different laboratory animals *in vitro* and *in vivo* The results conflict widely ranging from positive findings from sera of all patients to complete failure to demonstrate any difference between normal and myasthenic patients BERGH (1953) pointed out that the experimental procedures chosen by many investigators were such as to favour negative results NASTUK STRAUSS and OSSERMAN (1959) were unsuccessful in finding a circulating substance which inhibited neuromuscular transmission but found that sera obtained from some patients with myasthenia gravis possessed a cytolytic activity on muscle also occasionally exhibited by sera from apparently normal persons These authors pointed out that the possibility remained that symptoms of neuromuscular blockade could persist in the absence of the agent which originally produced the junctional derangement and which may have been present and active in the body only over short periods of time

More direct estimates of the sensitivity of myasthenic muscles to acetylcholine were obtained by close intra arterial injection of the drug LANARI (1937) and HARVEY and LILIENTHAL (1941) reported that myasthenic patients reacted with powerful muscle contractions whereas normal persons became paralyzed when 20—40 mg acetylcholine was used ACHESON LANGOHR and STANBURY (1948) BUCHTHAL and ENGBAEK (1948) and ENGBAEK (1950) determined the

minimal active dose of acetylcholine (less than 1 mg) necessary to elicit muscle contraction The latter authors found that higher than normal doses were necessary in myasthenic subjects whereas ACHESON *et al* (1948) found no difference

By combining electromyographic techniques and administration of different drugs such as curare decamethonium and acetylcholine to patients with myasthenia gravis several investigators have endeavoured to evaluate more closely the nature of the neuromuscular blockade in myasthenia gravis In a series of papers GROB and his coworkers (GROB JOHNS and HARVEY 1956 a b c JOHNS GROB and HARVEY 1956) compared the results obtained from normal subjects with those from myasthenic patients They found great similarities between the electromyographic responses to nerve stimulation in lightly curarized normal persons and in patients with myasthenia gravis There was a slight degree of block with single nerve stimuli and with repetitive stimulation there was an increase in the block as the interval between stimuli was reduced Following a period of tetanic nerve stimulation a progressive increase in the degree of blockade was followed by a period of facilitation after which the block continued to increase for several minutes The magnitude of these effects increased with the frequency and duration of the tetanus

By testing the effect of intra arterially injected acetylcholine on the electromyographic response to nerve stimulation GROB and coworkers (JOHNS *et al* 1956) found that after an initial prompt depression, proportionally smaller in myasthenics than in normals there was a period of transient decrease in the degree of blockade and in myasthenic patients even a facilitation of transmission which was then followed by late depression of the responses During this late period neostigmine improved transmission in myasthenic patients but had the opposite effect in non myasthenics CHURCHILL DAVIDSON and RICHARDSON (1953) obtained similar results with decamethonium

On the basis of these experiments GROB *et al* concluded that the defect in neuromuscular transmission in myasthenia gravis was produced by normally liberated acetylcholine which made an abnormal form of receptor substance insensitive to acetylcholine

In 1901 LAQUER and WEIGERT first described the relationship of thymic tumor to myasthenia gravis and in 1917 BELL drew attention to the high incidence of hyperplasia of the thymus in myasthenic patients BLALOCK HARVEY FORD and LILIENTHAL in 1941 reported great improvement in myasthenic patients after removal of the thymus gland Since then thymectomies have been carried out on many myasthenic patients in several large clinics The value of thymectomy in myasthenia gravis has not been conclusively demonstrated VIETS and SCHWAB (1960) reviewed the problem and recommended thymectomy only in patients not doing well on medical management and for whom the chance for spontaneous remission appeared small The results of thymectomy were most favourable in young female patients

The observations that the thymus in some unknown way may be involved in

myasthenia gravis and the accumulating evidence that the thymus is of great importance for antibody formation (BURNET 1962) led several investigators to suggest that myasthenia gravis is an autoimmune disease The common finding of lymphorrhages in skeletal muscle from myasthenic patients was used to strengthen this idea although RUSSELL (1953) considered them nonspecific

NASTUK *et al* (1959) found that serum from myasthenic patients more often possessed cytolytic activity on frog muscle than did control sera This led them to study serum complement activity and to look for a complement fixing immunologic system in patients with myasthenia gravis They found that serum complement levels varied considerably beyond both the upper and lower limits of normal (NASTUK PLESCIA and OSSERMAN 1960) By applying immunofluorescence techniques they were able to demonstrate the presence of a muscle binding complement fixing component not present in normal samples in a crude globulin fraction of sera from myasthenic patients This globulin was bound in striations on the muscle fibre in a rather discrete manner (STRAUSS SEEGAL HSU BURKHOLDER NASTUK and OSSERMAN 1960)

These experiments were confirmed by VAN DER GELD FELTKAMP and OOSTERHUIS (1964) and by BEUTNER WITEBSKY RICKEN and ADLER (1962) The latter authors summarized the evidence favouring the view that myasthenia gravis is an autoimmune disease as follows i) Autoantibodies appear to be associated with clinical manifestations but with present techniques they cannot be demonstrated in all cases ii) Passive transfer appears to occur in pregnancies iii) There is evidence in the literature suggestive of a possible direct effect of some myasthenic sera on contractions of isolated muscle iv) A histopathological finding compatible with an autoimmune disease occurs in the form of lymphorrhages in most fatal cases of myasthenia gravis

GROB HIMEI and NAMBA (1964) have isolated from normal and myasthenic skeletal muscle a ribonucleoprotein by precipitation with d tubocurarine It combined strongly with acetylcholine and d tubocurarine About half of the myasthenic patients but no normal subjects had serum complement fixing antibodies to this ribonucleoprotein This antibody combined with the A bands nuclei and end plates of normal and myasthenic muscle and also with nuclei of several other tissues Immunization of rabbits with a ribonucleoprotein obtained from normal human muscle produced antibodies with properties similar to the antibody found in the serum from myasthenic patients

Whether myasthenia gravis is an autoimmune disease in the sense that the muscle weakness is caused by an autoimmune process or whether the autoimmune phenomena observed are secondary has not yet been ascertained

PRESENT INVESTIGATIONS

METHODS AND MATERIALS

The methods used in this series of experiments have been presented in detail in the individual papers and therefore only a very short survey is included

Experiments have been performed *in vitro* on isolated nerve muscle preparations bathed in a modified Tyrode s solution. The preparations used were rat diaphragms and human intercostal muscles The control human muscles were obtained from patients without known muscular or neuromuscular disease undergoing thoracotomy the specimens from patients with myasthenia gravis were removed as a biopsy under regional anesthesia or at thymectomy

When rat diaphragm preparations were used, stimulation of the motor nerve was accomplished with ordinary bipolar electrodes As intramuscular nerve twigs had to be stimulated when human muscle was used smaller stimulating electrodes had to be employed Initially a micropipette filled with NaCl was used In later experiments fine bipolar platinum or tungsten electrodes insulated to the tip with glass were found more suitable

The conventional techniques for intracellular recording with KCl filled glass capillary microelectrodes mounted on micromanipulators were used The potentials were recorded by photographing oscilloscope traces and in later studies directly on paper

The clinical features of the patients with myasthenia gravis are summarized in table I The material for this table has been obtained from the patients case histories and hospital charts The patients are listed according to the order in which their muscles were investigated The first six patients were included in the initial (Paper II) and the remainder in the final study (Paper IV) Muscles from one patient no 4 were studied in both investigations She had had a thymectomy followed by partial remission 3 years prior to the first biopsy The remission was still present at the time of the second biopsy The results obtained at the two occasions were comparable None of the patients had had a complete remission prior to the biopsy but several had had partial remissions Muscle specimens from patients no 7 and 13 were obtained at thymectomy Patient no 11 who only had ocular symptoms at the time of biopsy developed severe generalized weakness two weeks later

In the table the symptoms refer to the muscle groups clinically involved in the disease Under the column headed *maximal* all the muscle groups which had shown symptoms up to the day of biopsy are listed This does not necessarily indicate that all the symptoms were present simultaneously In the column headed *present* the symptoms at the time of biopsy are given The symbols

TABLE I The clinical characteristics of the patients with myasthenia gravis

Patient no.	Sex	Age years	Duration of disease years	Clinical symptoms: Maximal	Clinical symptoms: Present	Remissions no.	Present medication: Drugs* and daily doses	Present medication: Effect
1	F	18	3	o b g r	o b g r	0	Neo 15 mg × 20 Mes 60 mg × 13–14	++
2	F	54	7	o b g r	o b g	2	Neo 15 mg × 4 Mes 180 mg × 5	++
3	M	57	11	o b g r	o g	2	Mes 60 mg × 10–15	++
4	F	30	5	o b g	o b g	1	Mes 180 mg × 7	+++
5	F	38	12	o b g	o b	1	Myt 10 mg × 6	++
6	F	41	18	o b g r	o b g	1	Neo 15 mg × 5–6	+
7	F	21	3	o b g r	o b g	1	Neo 30 mg × 7 Mes 60 mg × 2	++
8	M	43	15	o b g r	o b g r	0	Neo 15 mg × 6 Mes 30 mg × 6	++
9	M	46	1	o b g	o b g	0	Neo 15 mg × 8 Mes 60 mg × 3	+++
4	F	33	8	o b g	o b	1	Mes 60 mg × 4–5	++
10	M	45	2	o b g	o	1	none	
11	M	38	0 1	o	o	0	Neo 30 mg × 6 Mes 60 mg × 2	+++
12	M	32	6	o g	o g	0	Neo 15 mg × 5	+++
13	F	20	1	o b g r	o b g r	0	Neo 30 mg × 5 Mes 60 mg × 3	++

F = female M = male

* Neo = Neostigmine Mes = Mestinon® (Pyridostigmin NFN INN)
Myt. = Mytelase® (Ambenonium INN Ambestigmin NFN)

o b g and r refer to ocular bulbar generalized but without respiratory involvement and respiratory manifestations respectively

The effect of the medication was estimated on a five graded scale where 0 means no observable effect, + observable but clinically insignificant effect ++ readily observable improvement of function +++ marked but incomplete restoration of muscle strength and ++++ restoration of muscle function to normal

RESULTS

The resting normal human intercostal neuromuscular junction (Paper I)

For the study of human neuromuscular transmission *in vitro* it was necessary to find a muscle which could be removed from the body with both insertions intact and without harm to the patients The human intercostal muscle when properly dissected into small bundles fulfilled these requirements (DILLON FIELDS GUMAS JENDEN and TAYLOR 1955)

It was possible to obtain from the preparations good resting membrane potentials between 70 and 90 mV with a mean of about 80 mV The muscle bundles remained in good condition without any decay of resting potentials At the visually located end plate regions spontaneous depolarizations with amplitudes and time courses similar to the miniature end plate potentials recorded at other neuromuscular junctions were found They appeared to occur at random intervals Their frequency was somewhat lower than the frequency reported from other species and rates increased with increasing osmolarity of the bathing solution

The passive electrical membrane characteristics of the muscle fibres estimated by the method of square pulse analysis (BOYD and MARTIN 1959) was of the same order of magnitude as reported for other species

It appeared that the electrophysiological properties of this muscle were similar to those of other mammalian species and that the preparation was suitable for studying neuromuscular transmission in more detail and for evaluation of neuromuscular disorders such as myasthenia gravis

Myasthenia gravis I (Paper II)

Muscle specimens were obtained from six patients with myasthenia gravis and control muscles were obtained at thoracotomies in patients free of neuro muscular symptoms

In the myasthenic specimens end plate regions were localized visually and by the recording of end plate potentials with a fast risetime or of responses to iontophoretic microapplication of acetylcholine At most end plates no spon taneous activity in the form of miniature end plate potentials could be recorded In a few fibres miniature end plate potentials of about normal amplitude and frequency were found in others only an occasional spontaneous potential was recorded At normal human neuromuscular junctions as in other species an increase in extracellular potassium ion concentration resulted in a great increase in the frequency of the miniature end plate potentials In muscles from my

asthenic patients no miniature end plate potentials were detected after the potassium concentration was raised.

In the experiments where the end plate regions were localized by iontophoretic microapplication of acetylcholine the responses were of normal magnitude and the area of chemosensitivity was about 0 1–0 2 mm in both normal and myasthenic muscle fibres

The finding of normal responses to the iontophoretically applied acetylcholine and the recording in some fibres of miniature end plate potentials of about normal amplitude suggested that the postsynaptic chemosensitivity was not seriously reduced The failure to find miniature end plate potentials which responded with a high frequency discharge in raised potassium ion concentration was interpreted as the result of some defect in the release of acetylcholine from the motor nerve terminals This idea was strengthened further by a few experiments in which end plate potentials were recorded during and after a high frequency tetanus When the myasthenic preparations were stimulated indirectly through the nerve it was observed that neuromuscular transmission became blocked by a period of high frequency stimulation It must be noted that in control preparations tubocurarine was required to block transmission The result demonstrated a difference between the behaviour of myasthenic and curarized normal neuromuscular junctions during and after a high frequency tetanus At myasthenic junctions no or slight depression of the end plate potentials occurred during the tetanus and post tetanic potentiation was absent or small whereas these phenomena were both present in curarized normal preparations

Hemicholinium (Paper III)

From the results of the experiments described in the previous section it was suggested that in myasthenia gravis there was a prejunctional deficiency affecting either transmitter formation or release mechanisms It was therefore of interest to apply experimental procedures which could be expected to reduce transmitter formation or release at the neuromuscular junction

In 1955 SCHUELER introduced the drug hemicholinium no 3 (HC-3) which blocks the synthesis of acetylcholine in intact nervous tissue The drug also has been shown to cause a neuromuscular blockade With low doses of HC-3 the blockade was delayed in onset and occurred only if the motor nerve was stimulated repetitively for some time Higher doses caused a prompt neuromuscular block which was independent of the intensity of nerve stimulation The latter effect was clearly postsynaptic and investigations using intracellular recording from the motor end plates have shown only this curare or procaine like postsynaptic effect of the drug (see review by SCHUELER 1960) DESMEDT (1957) observed similarities between the neuromuscular block produced in cats treated with HC-3 and in patients with myasthenia gravis In the sympathetic ganglion BIRKS and MCINTOSH (1961) have shown that the release of acetylcholine declines as the acetylcholine content of the ganglion becomes reduced after the synthesis has been blocked by HC-3

It was therefore of interest to study the effect of HC-3 at the neuromuscular junction Experiments were performed in the rat diaphragm preparation When the phrenic nerve was stimulated repetitively the amplitudes of both the end plate potentials and the miniature end plate potentials were reduced as the stimulation was prolonged No such progressive reduction of the amplitude of the miniature end plate potentials was evident if the nerve was not stimulated to release acetylcholine The same phenomenon could be elicited whether trans mitter release was induced by electrical nerve stimulation of preparations blocked by tubocurarine or increased magnesium ion concentration or whether the release was caused by an increase in the external potassium ion concentration

The time course of the reduction in the amplitudes of the end plate potentials and the miniature end plate potentials and the finding of an unaltered post synaptic sensitivity to bath applied carbachol indicated that these were pre synaptic phenomena The amount of transmitter liberated in each acetylcholine quantum was reduced as stimulation of transmitter release was prolonged By analogy with the experiments on cat sympathetic ganglia it was concluded that the amount of acetylcholine liberated in each quantum was reduced as the presynaptic store of transmitter became depleted by stimulation after the block of acetylcholine synthesis with HC-3 had been introduced

On this hypothesis the total amount of releaseable acetylcholine in the motor nerve terminals could be estimated as the sum of all end plate potentials and/or miniature end plate potentials that could be produced after the acetylcholine synthesis was blocked by HC-3 As this value was dependent not only on the amount of acetylcholine released but also on the postsynaptic sensitivity a cor rection for the latter factor was introduced By dividing the total amount of end plate depolarization by the amplitude of the end plate depolarization produced by one acetylcholine quantum at the start of stimulation the estimate was expressed as number of quanta of initial size independent of the post synaptic responsiveness The amount of releaseable acetylcholine in a motor nerve terminal was estimated to be equivalent to about 250 000 quanta of initial size

Myasthenia gravis II (Paper IV)

With a method now available to reduce the amount of acetylcholine in a quantum and to estimate the acetylcholine content of the motor nerve terminals neuromuscular transmission in myasthenia gravis was studied more closely The study was undertaken primarily to determine whether the marked reduction in the amplitude of the end plate potentials was the result of a reduction in the number or in the size of the quantal components of the end plate potentials

In this study muscles were obtained from eight patients with clinically diagnosed myasthenia gravis Although the patients studied varied widely in the severity extent of involvement and duration of the disease (table I) no qualitative or quantitative differences in the behaviour of the intercostal neuro-

muscular junctions were observed The results from all patients were therefore evaluated as a group unless otherwise noted As in the previous study on myasthenic muscle (paper II) it was found that *in vitro* neuromuscular transmission rapidly became blocked when the motor nerves were stimulated repetitively at frequencies above 2–5/sec Spontaneous junctional activity could not be found at most end plates but when recording conditions were exceptionally good high amplification records often showed miniature end plate potentials In those fibres in which miniature end plate potentials were found the mean resting frequency was 0 22/sec the same as the frequency of miniature end plate potentials in normal human intercostal muscle Their amplitude was on the average only about one fifth of the amplitude of normal miniature end plate potentials During and after tetanic indirect stimulation and on raising the external potassium ion concentration there was an increase in their frequency

At junctions in which end plate potentials and miniature end plate potentials were both recorded calculations of the size of the quantal components of the end plate potentials gave values which corresponded to the size of the miniature end plate potentials It was assumed that the number of quanta in the end plate potentials were distributed according to Poisson s theorem (DEL CASTILLO and KATZ 1954 a) These data suggested that the myasthenic end plate potentials were composed of quanta which could also be manifest as miniature end plate potentials

The end plate regions of myasthenic and normal muscle fibres were depolarized to the same extent by bath application of decamethonium and of carbachol at two different concentrations It seemed therefore more likely that the quanta recorded at myasthenic junctions were small because less acetylcholine was released from the motor nerve terminals than that the postjunctional sensitivity to acetylcholine was reduced The possibility that normal amounts of acetylcholine were released from the motor nerve terminals but were destroyed by abnormally active cholinesterase was excluded by the failure of cholinesterase inhibitors to restore to normal the amplitude of myasthenic miniature end plate potentials

The number of quanta in the end plate potentials (quantum content) was estimated during different frequencies of repetitive nerve stimulation and no differences were found between myasthenic and normal junctions Manipulations known to change the quantum content such as post tetanic potentiation and changes in the external calcium and magnesium ion concentrations had the same effects as in normals

Estimations of the store of acetylcholine by the use of HC-3 as described in paper III gave values corresponding to about 200 000 quanta of initial size in both myasthenic and normal motor nerve terminals This observation indicated that if the quanta were small because of a deficiency of acetylcholine content then the absolute amount of releaseable acetylcholine in the nerve terminals was small in proportion If hemicholinium was not present in the

bathing medium there was no decline in quantum size when stimulation was prolonged and neither rest nor addition of choline restored it These findings indicated that the defect in quantum size in myasthenia was not due to an inhibition of acetylcholine synthesis similar to that produced by HC-3 Some of these preparations were vigorously washed in normal solution for several hours without any improvement of the neuromuscular defect showing that the neuromuscular block was not caused by an agent which could easily be washed away

The conflicting observations concerning miniature end plate potentials in paper II and IV were the result of improvement of the recording technique The noise level in the first study obscured the miniature end plate potentials observed in the second At some end plates in normal muscle with miniature end plate potentials about 1 mV in amplitude spontaneous potentials with an amplitude of 3–6 mV were observed These were abolished or greatly reduced in frequency when potassium was added They probably represented the simultaneous release of several acetylcholine quanta brougt about by injury of the nerve terminal They may be the counterpart of the spontaneous depolarizations recorded in study II at a few myasthenic end plates as the latter also disappeared when potassium was added They were mistaken for miniature end plate potentials as the true myasthenic miniature end plate potentials were submerged in the base line noise and as it was not known that the quanta could be deficient in their acetylcholine content

Release of acetylcholine during nerve stimulation (Paper V)

During the investigation of the neuromuscular transmission process in myasthenia gravis (paper IV) one of the problems encountered was to characterize normal quantitative behaviour of human end plate potentials under a variety of conditions A quantitative investigation of transmitter release at the neuromuscular junction was therefore undertaken in normal intercostal muscle

The methods for a detailed analysis of the release of transmitter from the motor nerve terminals during activity rely on intracellular recording of the post synaptic responses elicited by the transmitter To perform this type of recording the muscle cannot be allowed to twitch in response to nerve stimulation For this purpose tubocurarine or magnesium are generally used to block neuromuscular transmission As magnesium causes neuromuscular block by reducing the amount of transmitter that is released per impulse it was not a suitable agent for the study of the amounts of transmitter normally liberated by a nerve impulse Tubocurarine blocks neuromuscular transmission by reducing the post synaptic sensitivity to acetylcholine With a completely paralyzing dose of tubocurarine the postsynaptic sensitivity was so depressed that miniature end plate potentials could not be seen However in lightly curarized preparations it was possible during repetitive stimulation to record miniature end plate potentials and end plate potentials at the same junction In such experiments it was shown that quantum size could be estimated from the amplitude of the miniature

end plate potentials as well as from a statistical analysis of the amplitude distribution of sequential end plate potentials The two independent methods gave corresponding values for quantum size On this basis quantum size was estimated in other experiments in which so much tubocurarine had to be used that no miniature end plate potentials could be observed

It is well known that the end plate potentials decline in the beginning of a tetanus and more so as the frequency of stimulation is increased After the first few impulses the height of the end plate potentials remained fairly constant By estimating quantum size at single junctions at different frequencies of stimulation it was shown that this decline in the amplitude of the end plate potentials was caused by a reduction in their quantum content If the end plate potentials were reduced in height during tetani only because their quantum content changed the quantum content of any end plate potential at a junction could be taken as the amplitude of the end plate potential divided by quatum size

Calcium increased the quantum content of the end plate potentials at low frequencies of stimulation and at the onset of tetani when high frequencies (above about 50/sec) of stimulation were used The decline in quantum content in the beginning of a tetanus was accelerated by calcium When high stimulation frequencies were used calcium did not change the quantum content of the end plate potentials after the first few impulses The effect of magnesium was essentially the opposite and again was evident only on the first few impulses when the stimulation frequency was high

When the quantum content of sequential end plate potentials in a tetanus was plotted against the accumulated output of quanta there was a virtually linear fall of the first few end plate potentials This observation was consistent with the concept that the rundown of the amplitude of the end plate potentials in the beginning of a tetanus reflected depletion of a presynaptic store of transmitter immediately available for release (LILEY and NORTH 1953 OTSUKA ENDO and NONOMURA 1962) The extrapolation of this straight line to the abscissa gave an estimate of this store amounting to 300–1000 quanta The effects of calcium and magnesium were to increase and decrease respectively the fraction of this store released by an impulse without affecting the size of the store

In a similar way the effect of a conditioning tetanus on the release of transmitter by a second tetanus was investigated It was found that after the conditioning tetanus the store of transmitter immediately available for release was reduced and the fraction of it which was released per impulse increased

After the first few impulses the quantum content of the end plate potentials remained relatively constant The rate at which transmitter was liberated after the initial fall increased with increased frequency of stimulation to a maximum at about 70/sec At this and higher frequencies about 2500 quanta were released per second If the stimulation was continued for long periods of time there was a further gradual decline in the amplitude of the end plate potentials Finally when the quantum content of the end plate potentials had re

very low level they ceased to decline further A graph of the amplitude of the end plate potentials against the accumulated sum of the previous end plate potentials gave a straight line when allowance had been made for the level at which the end plate potentials finally remained This result was consistent with the hypothesis that the slow rundown of the amplitude of the end plate potentials during prolonged stimulation reflected depletion of a presynaptic store of transmitter The size of this store was estimated to be about 25 000 quanta This is about 10 % of the total releaseable acetylcholine present in the motor nerve terminal (paper III)

Release of acetylcholine by potassium stimulation (Paper VI)

The emptying of stores of transmitter in the nerve terminal and the repletion of these when repetitive nerve stimulation was given were compared with the kinetics of rapid release of quanta recorded as miniature end plate potentials produced by depolarization with potassium ions

A potassium ion concentration of 30 mM caused release of acetylcholine quanta at a rate of about 150/sec at human intercostal neuromuscular junctions The increased frequency produced by this and lower concentrations of potassium was well maintained for at least six hours As in the rat diaphragm (LILEY 1956 c) the logarithm of the frequency was related in a linear fashion to the logarithm of the potassium ion concentration If the potassium ion concentration was raised to 40 or 50 mM an initially greater increase in frequency was followed by a marked reduction often approaching the resting frequency

The decline in rates of release of acetylcholine quanta was not produced simply by the presence of the very high potassium ion concentration as acetylcholine quanta continued to be released if the rates produced by 40 or 50 mM potassium were restricted to an upper limit of about 150 quanta/sec by altering calcium and magnesium ion concentrations The total number of acetylcholine quanta which were released by 40 or 50 mM potassium and normal calcium magnesium levels averaged about 220 000 This quantity was similar to the amount of acetylcholine which was released after the synthesis of acetylcholine had been blocked by HC-3 (papers III and IV)

DISCUSSION

Storage and release of acetylcholine at the normal neuromuscular junction

The studies of the dynamics of acetylcholine release at the neuromuscular junction (papers III V and VI) provide a basis for a hypothetical model of how transmitter is manufactured stored and made available for release in the motor nerve terminals

The transmitter released by a nerve impulse is derived from a presynaptic store of transmitter immediately available for release containing normally about 300–1000 quanta presumably already packaged as such The actual number of quanta released by an impulse is governed by two factors i) the amount in the store and ii) the probability of release of each quantum in this store Calcium magnesium and post tetanic potentiation apparently affect mainly the probability of quantal liberation Neither calcium nor magnesium influenced the size of this store and the effect of a conditioning tetanus was to deplete it

The liberation of transmitter from this store results in its depletion It is only because transmitter becomes mobilized into it that the nerve terminals can *continue to release transmitter The rate of mobilization is apparently governed to a large degree by depletion of the immediately available store but is also dependent upon the frequency of stimulation and may be delayed in onset* This observation is consistent with those of HUBBARD and WILLIS (1962) which indicated that hyperpolarization of the motor nerve terminals caused an increased mobilization of transmitter which was slow in onset Such a hyperpolarization was normally seen during repetitive high frequency stimulation (HUBBARD and SCHMIDT 1963)

The continued decline of the amplitude of the end plate potentials with continued stimulation is an indication that the presynaptic store from which transmitter is derived has a limited size and is not replenished as fast as it can be emptied The size of this store was estimated to be about 25 000 quanta The content of releaseable transmitter in human intercostal motor nerve endings is considerably greater equivalent to about 200 000 quanta

Experiments using perfused sympathetic ganglia have revealed the existence of two rather distinct presynaptic pools of transmitter a readily releaseable fraction which is partially depleted at moderate or high rates of nerve stimulation and a non readily releaseable fraction which is maintained at a constant level unless synthesis of acetylcholine is inhibited by hemicholinium or choline deficiency (BIRKS and MACINTOSH 1961) It is likely that the store from which transmitter is mobilized to become available for release for convenience called

mobilization store corresponds to the readily releaseable fraction of BIRKS and MACINTOSH The remainder of the releaseable presynaptic pool corresponds to their non readily releaseable fraction

The effect of hemicholinium at the neuromuscular junction is solely on quantum size suggesting that the non-readily releaseable store in the nerve terminal consists of transmitter or possibly transmitter precursor which is not already in the form of quanta This store represents a reservoir of transmitter from which quanta can be formed making the transmitter available for mobilization and subsequent release The quanta become deficient in their acetylcholine content when this store is depleted by stimulation of transmitter release in the presence of hemicholinium block of acetylcholine synthesis The amount of acetylcholine in a quantum therefore appears to be governed by the amount or concentration of acetylcholine in this store ECCLES (1964) states "It seems likely that intense stimulation of transmitter release in the presence of HC-3 is the first experimental procedure by which it has been possibly to modify the amount of acetylcholine in a quantum" The mechanism by which hemicholinium blocks the synthesis is not revealed by these experiments It may block the uptake of choline by the motor nerve terminal or hemicholinium may in itself be taken up and then dealt with as a false transmitter

The experiments with the intense potassium stimulation of acetylcholine release gave the unexpected result that the release of acetylcholine quanta practically stopped after an amount equal to the total releaseable acetylcholine content of the terminals had been released This correlation may be largely fortuitous but the possibility that the very high release rates produced by 40 or 50 mM potassium may have interfered with the formation or release of acetylcholine quanta must be considered There are several alternative models i) a pool of an unknown substance necessary for quantal formation or release becomes depleted by the very high release rates ii) acetylcholine quanta cannot be released unless full and the synthesis of acetylcholine cannot keep up with the very high release rates so that the non readily releaseable fraction becomes depleted The latter hypothesis requires that in the hemicholinium experiments this drug substitutes for acetylcholine in the quanta thus making them releaseable

Although the experimental results are fully compatible with the suggested overall model they do not prove it Other models can be suggested which fit as well for example the nerve terminal contains no acetylcholine in the form of quanta but quanta are formed only during the process of transmitter liberation All the phenomena ascribed here to depletion of the immediately available store could then be attributed just as well to exhaustion of the ability of the nerve terminal membrane to form quanta Mobilization could merely reflect the rate at which some material was made available for use in the release process A store of such material would correspond to the mobilization store with a slow rate of renewed synthesis

end plate treats as a transmitter (false transmitter) Similar mechanisms may operate if the acetylcholine is not stored as quanta but quantal packets are formed in the process of release

The question arises whether the suggested presynaptic defect can explain the clinical characteristics of myasthenic neuromuscular transmission and the effect of certain drugs upon it

A typical phenomenon of neuromuscular transmission in the beginning of a tetanus is the decline in amplitude of sequential end plate potentials At normal neuromuscular junctions the end plate potentials have a great safety factor and despite their rundown in size remain suprathreshold and no neuromuscular blockade occurs However at myasthenic junctions the first end plate potential may be close to threshold due to the small amounts of acetylcholine in the quanta and the subsequent decline in the amplitude of the end plate potentials is often so great that the end plate potentials become subthreshold and a neuromuscular blockade is produced Even the first end plate potential may in some fibres be subthreshold because of this fact such facilitory phenomena as post tetanic potentiation can result in temporary improvement in the overall performance of a muscle in myasthenia gravis In normal muscle with suprathreshold end plate potentials facilitating events at the neuromuscular junctions will not be observed in the gross performance of the muscle

The cholinesterase inhibiting drugs prevent the enzymatic hydrolysis of acetylcholine allowing the transmitter to react longer at a higher concentration and therefore at a larger area of the muscle end plate The duration and the amplitude of the end plate potentials become increased and the end plate potentials in a tetanus may sum up improving impaired neuromuscular transmission The supersensitivity of myasthenic patients to tubocurarine is to be expected if a smaller than normal amount of acetylcholine is liberated

A common finding is that myasthenic patients are less sensitive to the neuromuscular blocking properties of the so called depolarizing agents decamethonium and succinylcholine It is well known that this type of drug has two main effects at the muscle end plates depolarization but also reduction in the chemosensitivity of the acetylcholine receptors presumably in a non competitive manner often called desensitization The desensitization dominates at high drug concentrations (THESLEFF 1955 GISSEN and NASTUK 1965 ROBERTS and THESLEFF 1965) Large depolarizations of the motor end plate cause a neuromuscular blockade whereas smaller depolarizations facilitate impaired neuromuscular transmission by reducing the minimum amplitude of an end plate potential necessary to reach the threshold for triggering an action potential in the muscle fibre (GISSEN and NASTUK 1964) Receptor desensitization reduces the amplitude of the end plate potentials but it is likely [illegible] at the dose levels used in humans the facilitory action su[illegible] zation In normal muscle transmission is maximal to beg[illegible] can only depress transmission

The difference in response of [illegible] intra arterial

injection of acetylcholine described by GROB JOHNS and HARVEY (1956 b c) may also be explained in a similar way GROB *et al* found that during the period of late depression after the injection of acetylcholine prostigmine improved transmission in myasthenic subjects but had no or a depressing effect in normal persons They interpreted this finding as showing an altered response of myasthenic end plates to acetylcholine It seems however more likely that this late effect of acetylcholine is presynaptic in origin resulting in a blockade of the motor nerve terminals (ARMETT and RITCHIE 1960 ROBERTS and THESLEFF 1965) In the records of GROB *et al* they have plotted the amplitude of the first and the fourth electromyographic response to a train of nerve stimuli one would expect that, if a block of neuromuscular transmission due to post synaptic phenomena is produced the fourth response should be reduced more than the first owing to the fact that the amplitude of the end plate potentials declines in the beginning of a tetanus This is what was observed in myasthenic patients and when tubocurarine was used in normals During the late phase of the acetylcholine block in normal persons no such dissociation of the first and fourth responses occurred indicating that this block was presynaptic in origin Under these circumstances it is to be expected that prostigmine was ineffective

DESMEDT (1957 1958) has reported similarities between the myasthenic block and that produced in cats treated with hemicholinium particularly with regard to the post tetanic exhaustion As pointed out previously there are differences between the small quanta formed in myasthenia and those found after stimulation of transmitter release in the presence of hemicholinium The phenomenon of post tetanic exhaustion may be attributed to normal depletion of the presynaptic stores of acetylcholine resulting in reduced amplitude of the end plate potentials and further impairment of transmission

It is concluded that small quantum size suffices to explain the clinical and pharmacological observations which have been made in myasthenia gravis The relationship of the autoimmune phenomena associated with myasthenia gravis to the symptoms of the disorder is not clarified by these studies

In most patients the intercostal muscles were not clinically involved at the time the specimens were obtained The typical defect was nevertheless found to a similar degree in all patients The resolution afforded by the techniques of clinical and experimental observations may have been insufficient to reveal differences between the patients nonetheless the pathological process appears to be generalized The clinical manifestation of selective involvement and of fluctuating intensity may reflect temporal and local variation in the intensity of the defect or in the safety factors involved in neuromuscular transmission.

CONCLUSIONS

The concept that transmitter is released from the motor nerve terminals in the form of acetylcholine quanta has contributed greatly to our present knowledge of synaptic transmission It has provided the basis for the performance and discussion of these studies

The experiments with hemicholinium however have revealed that the acetyl choline quantum is not as invariable in size as was previously believed but can be altered by experimental procedures

Investigations with repetitive stimulation of the normal neuromuscular junc tion have shown several weak links in the chain of events leading to the release of acetylcholine quanta i) The pool from which acetylcholine quanta is directly released is only sufficient for a few impulses ii) The fraction of this pool which is released by a nerve impulse is influenced by the ionic environment and by previous activity iii) The rate at which transmitter release can continue with repetitive stimulation is governed by the mobilization of acetylcholine quanta into this pool iv) The store of transmitter from which acetylcholine quanta are mobilized to become available for release has a limited size and is only slowly replenished probably by the formation of new quantal units Mobilization can therefore only keep up with intense release for a short period of continuous rapid stimulation v) The synthesis of acetylcholine in the nerve terminal can be insufficient due to the lack of substrate or due to the presence of inhibiting drugs such as hemicholinium and acetylcholine content of the quantal packets may be reduced

Studies of the neuromuscular transmission in myasthenia gravis demonstrated that this series of events can be affected by pathological conditions The results indicate that in myasthenia gravis there is a deficiency in the amount of acetyl choline in the transmitter quantum probably brought about by a defect in the quantum formation mechanism or by the presence of a false transmitter

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The circadian rhythm of self-selected rest and activity in the canary and the effects of barbiturates, reserpine, monoamine oxidase inhibitors and enforced dark periods

BY

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fil. lic. Robert N. Elston B. Sc. M. I. Biol.

Chapter V is based upon the present results (I) and those of two earlier papers:

Wahlström 1964 b (II)
Wahlström 1964 c (III)

Uppsala 1965
Appelbergs Boktryckeri AB

CONTENTS

INTRODUCTION

In recent years there has been a steady increase of interest in investigations dealing with biological clocks and the related phenomen of rhythmic changes between sleep and wakefullness The general field of biological clocks has been surveyed in several monographs (Cloudsley Thompson 1961 Bünning 1963 Harker 1964) reviews (Harker 1958 Webb and Brown 1959 Aschoff 1963) and symposia (1960 1962 1964 and 1965 references part 1) The relationship between biological clocks and navigation has been reviewed by Kalmus (1964) The clinically important field of sleep and wakefullness is covered by the new edition of Kleitman s classic work (1963)

In the canary (*Serinus canarius*) a circadian rhythm (Halberg 1959) becomes apparent when the birds have the choice between light and darkness and thus can chose when they want to be active and when they want to rest (Wahlström 1964 b) This self selection technique is suitable for pharmacological purposes and for the investigation of how duration of activity and rest are connected with the underlying biological clock

In the present investigations the connection between the biological clock mechanism and the pattern of activity and rest have been studied by disrupting the self selection situation for a couple of hours by depriving the birds of light when they expected it In a previous paper a brief description of some results from such experiments has been given (Wahlstrom 1965)

The effects of reserpine on activity and rest has been reported earlier (Wahlstrom 1964 c) Further pharmacological studies with monoamine oxidase inhibitors and barbiturates will be reported in this paper A few of these experiments have been briefly discussed in a previous paper as examples of drug induced changes in the self selected rhythm (Wahlström 1964 a)

CHAPTER I

METHODS

1 General experimental method

Details regarding the method have been published elsewhere (Wahlström 1964 b) and only a brief description will be given here

The canaries (males if not otherwise stated) are kept singly in lightproof wooden cages which are equipped with two perches One perch (the day perch) is used only to record the activity of the bird when the lights is on in the cage The other perch (the night perch) regulates the light in the cage When the birds hop onto this perch the light is extinguished and when they leave it the light is turned on The canaries thus have the possibility to choose between light and darkness

The light is placed in a lamphouse behind the cage and consists of a common electric bulb The strength of the lamp was 75 W in all experiments except some of those with pentobarbital in which case (included in Fig 18) it was 40 W

Each perch actuated one pen on a multichannel event recorder (Esterline Angus) Paper speed was 1.5 inch/h The night perch was tested every time food and water was controlled

The canaries have had access to clam shells sand water and commercial canary seed mixture *ad libitum* Food and water was attended to if possible once every activity period Once a week the birds were weighed their general conditions specially noted and at the same time they always got hardboiled eggs and depending on the season also apples oranges or lettuce

The normal rhythm consists of one period of activity[1] and one of rest Added together they give the circadian period The circadian period has thus been calculated from one waking up time to the next

[1] In this paper the terms activity and rest refer to the *durations* of the respective periods If amount of activity in the more conventional sense is dealt with it will be called locomotor activity

one Sometimes the activity is divided into two or more parts by short intervals when the bird is sitting on the night perch Such intervals on the night perch shorter than 30 min have always been disregarded When the birds stayed on the night perch for 30 min or more during their normal activity period then the duration of activity can be calculated in two ways *Overall activity* (=rest periods of 30 min or more during the activity disregarded) has been used in the experiments with an enforced period of darkness (EPD) In these experiments the properties of the roosting which definitely ended the activity were of interest *Gross activity* (=rest periods of 30 min or more during the activity regarded as short rest periods and added to the rest period after the roosting which definitely ended the activity) have been used in the drug experiments In these experiments the changes in activity as compared to rest was of interest The frequency of divided activity periods and the relation between overall and gross activity have been discussed elsewhere (Wahlstrom 1964 b)

The shortest interval allowed between two succeeding experiments in the same bird with EPD s and barbiturates was one week with the *lower doses of phenipra̧zine unless otherwise stated three weeks with* the higher doses and with pargyline and nialamide four weeks

2 Drug experiments

In the weeks before and after a drug administration the birds have if possible been handled and given distilled water instead of the drug at approximately the same time in the circadian period and in the same manner (single blind experiments)

All drugs used in the experiments to be reported in chapter III and IV have been administered orally and have unless otherwise stated always been administered as a single dose The drugs in powder form were dissolved in distilled water immediately prior to administration The solutions were administered by a stomach tube (a small polyethylene tube fitted to a syringe) The volume administered was always 1 0 ml/100 g body weight The birds were observed for approximately 1 min after the administration to see that they really kept down the solution

The following substances have been used nialamide hydrochloride (Niamid[R] Pfizer) pargyline hydrochloride (Mo 911 Eutonyl[R] Abbott lab) phenipra̧zine hydrochloride (JB 516 Catran[R] Draco) sodium pen

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tobarbital and sodium barbital All doses refer to the form of the substance stated above

The drugs have been administered at three different times in the circadian period of the birds *AM* means that the drug was given during the first half of the activity *PM* that it was given during the second half of the activity and *N* that it was given during the rest period

If there was light in the cage for more than 15 min after an N administration this light time has been added to the activity prior to the rest period in which the drug was given

The birds were usually observed once or twice through a spy window or if no clear view of the bird could be obtained through the window by opening the door at irregular intervals after a drug administration These observations were performed more frequently if any change in the behaviour was recorded

3 Statistical methods

In all experiments if not otherwise stated the induced changes have been calculated as increases over the average of a pre experimental period consisting of the 5 circadian periods immediately prior to the experiments Each experiment has been treated as a statistical unit All statistical calculations have been performed according to Snedecor (1956) All probabilities have been based on the t distribution if not otherwise stated t values for regression and correlation coefficients have been calculated in the following manner For regression $t = b/s_b$ where b is the regression coefficient and

$$s_b = s_{y \cdot x} / \sqrt{\Sigma x^2}$$

$s_{y \cdot x}$ is derived from

$$s_{y \cdot x}^2 = (\Sigma y^2 - b^2 \Sigma x^2) / (n-2)$$

For correlation

$$t = r/s_r = r \sqrt{(n-2) / (1-r^2)}$$

where r is the correlation coefficient Σx^2 and Σy^2 = sums of squares n = number of experiments (The abbreviation s_r has not been used by Snedecor)

CHAPTER II

EXPERIMENTS WITH ENFORCED PERIODS OF DARKNESS

1 Influence of an enforced period of darkness at the beginning of the activity

a The phenomenon as a whole

A preliminary communication has been given earlier (Wahlström 1963) Some results have also been presented at a summer school on circadian rhythms (Wahlström 1965)

In the experiments to be described in this part an enforced period of darkness (EPD) has been imposed on the normal selfselected rhythm of the canaries The EPD reported here occurred over the first part of the activity It was arranged by disconnecting the lamp in the cage during the rest period Thus when the birds hopped from the night perch no light came on in the cage After different time intervals from this attempted waking up the lamp was connected again The length of the EPD is the time from the attempted waking up to the time when the lamp was connected again (W_1L in Fig 2)

In the majority of experiments with EPD s concomitant with the start of activity the birds left the night perch and hopped to the floor or to the day perch If they hopped to the day perch they moved about a little and then left for the floor after various time intervals The activity on the floor could not be recorded In some experiments the birds moved about on the night perch at waking up time but did not immediately leave it Movements occurred with approximately the same frequency as recorded from the day perch during corresponding times after attempted waking up After various time intervals the birds also in these cases ended up on the floor When the birds did not leave the night perch immediately the time of attempted waking up was taken as that of the first movement on the night perch This in almost every experiment occurred at the time expected from the pre experimental waking up

Three examples of the effects of EPD s of various lengths are shown in Fig 1 The EPD s were approximately 4 (day 914 exp A) 6 (day 825 exp B) and 9 hours (day 833 exp C) After these EPD s there

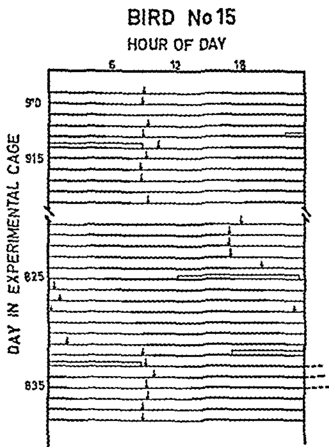


Fig 1 Examples of the changes induced by an enforced period of darkness Thin lines signify activity thick lines rest Arrows indicate administration of distilled water The second line on days 913—914 825 and 832—833 indicates the time during which the lamp was disconnected Date of day 915 25 July 1963

was in experiments A and B only a slight influence on the following roosting In experiment C the roosting was delayed by 3 hours The first waking up after the EPD was however much more influenced In these examples it was delayed by 2—3 hours After this first delay the pre experimental time relationship between two succeeding waking up times was re established The roostings showed different behaviours In experiments A and B some delay occurred in the first roosting after the EPD but two or three more roostings had to occur before the pre experimental time relationship to the waking up was re established and activity and rest had reached pre experimental length An LPD concomitant with the onset of the activity thus induces an immediate delay in the following waking up but no further change of it The

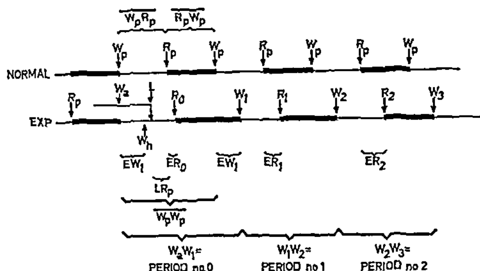


Fig 2 Schematical illustration of the effects of an enforced period of darkness (EPD) on the circadian rhythm The symbols are explained in their logical order

Pre experimental (Normal)

W_p = waking up

R_p = roosting

$\overline{W_pW_p}$ = average circadian period

$\overline{W_pR_p}$ = average activity

$\overline{R_pW_p}$ = average rest

Experimental (Exp)

W_a = attempted waking up No light Start of period no 0

L = return of light after EPD

LR_p = light up to expected roosting $\overline{W_pR_p} - W_aL$

R_0 = first roosting after EPD

ER_0 = delay of first roosting after EPD $W_aR_0 - \overline{W_pR_p}$

W_1 = first waking up after EPD Start of period no 1

EW_1 = delay of first waking up after EPD $W_aW_1 - \overline{W_pW_p}$

W_h = hypothetical waking up EW_1 hours after W_a

ER_1 and ER_2 are delays in roosting R_1 and R_2 calculated in the same manner as ER_0

roostings have a tendency to change more gradually to their pre experimental time relationship with the waking up times

These findings are schematically illustrated in Fig 2 This figure will be used in what follows as a reference to the different changes which will be further described The upper line is the pre experimental rhythm and the lower line shows the changes induced by an EPD (W_aL) The first roosting (R_0) is delayed but not as much as the first waking up (W_1) ER_0 is smaller than EW_1 There is no further change

in the waking up times after the first delay W_1W_2 and W_2W_3 are equal to $\overline{W_pW_p}$ The second roosting (R_1) appears closer to the time expected from the pre experimental rhythm than the waking up ER_1 is smaller than EW_1 The pre experimental rhythm is re established in circadian period no 2 ER_2 is equal to EW_1

In Fig 3 the induced delay in the first waking up after an EPD has been plotted against the length of the EPD The figure shows the data from all experiments performed with EPD s covering the onset of activity The delay (EW_1 in Fig 2) has been calculated as the difference between the length of the circadian period during which the EPD was given (the time between attempted waking up W_x and the next real waking up W_1) and the average length of the 5 circadian periods immediately prior to the EPD ($\overline{W_pW_p}$ in Fig 2) With longer EPD s (more than 2 hours) the delay is less than the EPD (the points fall below the 45° line) and there is a considerable variation With a short EPD (less than 2 hours) the delays are roughly equivalent to the length of the EPD (the averages follow the 45° line in Fig 3 see also Table I) The deviations from the line correspond to the varia

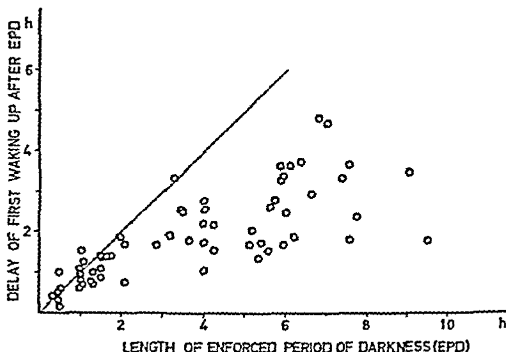


Fig 3 Influence of the length of the EPD on the delay obtained in the first waking up after the EPD (EW_1) All reported experiments with EPD s are included

tions in the length of the circadian period seen during normal conditions standard deviation 0 1—0 2 hours (Wahlström 1964 b)

The experiments performed with an EPD of less than 2 hours have been divided into three series Series $D_{0.5}$ consists of the experiments with an EPD of 0 5 (range 0 26—0 75) hours series D_1 with an EPD of 1 0 (0 76—1 25) hours and series $D_{1.5}$ with an EPD of 1 5 (1 26—1 75) hours The results are given in Table I Circadian period no 0 is the period starting with the attempted waking up The activity in circadian period no 0 (W_xR_0 Fig 2) has been calculated from the attempted waking up to the roosting The pre experimental average was calculated on the 5 circadian periods immediately prior to circadian period no 0

Table I shows that the length of circadian period no 0 is increased with the length of the EPD in series $D_{0.5}$ and D_1 In series $D_{1.5}$ there is a difference between the EPD and the increase in circadian period no 0 (0 02>P>0 01) An EDP of 1 5 hours is thus not totally compensated by the next waking up With few exceptions the lengths of the circadian periods after no 0 are not changed compared with the pre experimental average This means that a delay was induced in the waking up starting circadian period no 1 and after that waking up there was a pre experimental time relationship between the following waking ups ($\overline{W_pW_p}=W_1W=W W_3$ Fig 2)

In series $D_{0.5}$ and D_1 the increase in circadian period no 0 corresponded to the length of the EPD The time from the end of the EPD to the following waking up (LW_1 Fig 2) thus correspond to the length of the pre experimental circadian period In these two series with short EPD s it appears as if the end of the EPD was taken as a new starting point for the series of waking up times Thus it seems as if the light appearing after the EPD in these cases somehow started the clock responsible for measuring the time period to the next waking up A certain amount of light before the EPD should then be able to inhibit the effect of an EPD That this is so will be demonstrated in chapter II part 2

The change in the length of circadian period no 0 was not primarily due to an immediate delay in the first roosting following the EPD The change in activity in circadian period *no 0* did not correspond to the change in the length of the circadian period In all three series the roosting (R_0 Fig 2) appeared later than normal (recorded as an increase in the length of the activity in Table I) but this delay was smaller than the increase in the circadian period no 0

Table 1 Changes in activity, rest and circadian period after an LPD of less than 2 h. Changes calculated as increase over the average of the five circadian periods prior to the LPD

Variable	Series	Pre exp. av.		Number of birds[b]	n[a]	Duration of LPD		Increase over pre exp. average in period no.							
								0		1		2		3	
		h	± S.E.			h	(range)	h	± S.E.	h	± S.E.	h	± S.E.	h	± S.E.
Circadian period	$D_{0.5}$	23.9[illegible]	0.33	6	6	0.49	(0.33–0.56)	0.31	0.12	−0.06	0.09	0.20	0.08	0.01	0.05
	D_{1}	23.8[illegible]	0.13	6	10	1.04	(0.98–1.23)	0.96	0.09	0.02	0.09	0.[illegible]0	0.07	0.05	0.05
	$D_{1.5}$	23.88	0.[illegible]2	6	8	1.50	(1.30–1.75)	1.16	0.10	0.10	0.08	0.01	0.10	0.12	0.07
Activity	$D_{0.5}$	12.[illegible]	0.89	6	6	0.49	(0.33–0.56)	0.25	0.38	−0.24	0.26	0.41	0.62	−0.02	0.[illegible]4
	D_{1}	11.52	0.76	6	10	1.04	(0.98–1.[illegible]3)	0.33	0.14	−0.29	0.13	−0.05	0.12	−0.26	0.21
	$D_{1.5}$	11.10	0.67	6	8	1.50	(1.30–1.75)	0.71	0.28	−0.52	0.30	0.48	0.47	0.19	0.31
Rest	$D_{0.5}$	11.73	0.81	6	6	0.49	(0.33–0.56)	0.[illegible]6	0.27	0.17	0.18	−0.22	0.55	0.03	0.19
	D_{1}	12.33	0.76	6	10	1.04	(0.98–1.23)	0.63	0.14	0.30	0.16	0.25	0.15	0.31	0.23
	$D_{1.5}$	12.40	0.62	6	8	1.50	(1.30–1.75)	0.45	0.27	0.62	0.[illegible]6	−0.16	0.16	−0.08	0.27

a) n indicates number of experiments each counted as a statistical unit

b) one bird in each series was an established female

($LR_0 < EW_1$ Fig 2) A general trend towards a shorter activity and a longer rest in period no 1 can be seen There were no further common changes in the periods after no 0 and none of the changes reached 3 times the standard error

The results of experiments with EPD s longer than 3 hours are shown in Table II A The length of the circadian period no 0 is increased In the following periods the circadian period is of pre-experimental length The effect of the EPD on the length of the circadian period is thus obtained wholly in period no 0 by a delay of the waking up starting circadian period no 1 (EW_1 Fig 2)

The roosting in circadian period no 0 (R_0 Fig 2) is delayed but not as much as the following waking up (W_1 Fig 2) In Table II A this is shown by a smaller increase in activity as compared to the increase in the circadian period

In circadian periods nos 1 2 and 3 there was a shorter activity than in the pre experimental periods ($W_1P_1 < \overline{W_pP_p}$ $W_2P_2 < \overline{W_pP_p}$ and $W_3R_3 < \overline{W_pR_p}$) The rest periods ($P_1W_2$ P_2W_3 and P_3W_4) were correspondingly increased This means that the roosting tended to appear earlier after the waking up than in the pre experimental rhythm and at the same time closer to the time to be expected from the pre experimental roostings ($ER_1 < EW_1$ $ER_2 < EW_1$ and $ER_3 < EW_1$) In circadian period no 4 the activity and rest is of pre experimental length The roosting now appeared at the expected time in the circadian period ($ER_4 = EW_1$) The roostings thus change to their normal position within the circadian period but this is not accomplished until circadian period no 4 A tendency towards the same changes in circadian period no 1 in the experiments with short EPD s has already been pointed out

During periods 1—3 the decrease in activity was of the same magnitude (Table II A) The standard error was however gradually increasing This depends partly on the different number of periods in the individual experiments which had to pass before the pre experimental relation between activity and rest was re established and also on the 5 experiments which did not return to the pre experimental relationship between activity and rest at all within 5 circadian periods (Table II B) In the latter cases it could have been either an induced or a spontaneous change The relationship between activity and rest within the circadian period is not constant during normal conditions (Wahlström 1964 b)

The immediate change in the waking up times and the gradual

Table II 4 Changes in activity rest and circadian period after an FPD of more than 3 h Changes calculated as increase over the average of the five circadian periods prior to the FPD

Variable	Pre exp av		Number of birds	Increase over the pre experimental average in period no																	
				0			1			2			3			4			5		
	h	±SE		h	±SE	n	h	±SE	n	h	±SE	n	h	±SE	n	h	±SE	n	h	±SE	n
Circadian period	24 01	0 08	12*	2 58	0 15	36	0 00	0 07	36	0 00	0 06	36	−0 03	0 05	36	0 13	0 08	34	0 06	0 07	32
Activity	12 16	0 29	12	1 47	0 18	36	−0 65	0 10	36	−0 60	0 15	36	−0 62	0 19	36	0 08	0 26	34	−0 12	0 23	32
Rest	11 85	0 28	12	1 10	0 13	36	0 64	0 11	36	0 60	0 16	36	0 59	0 16	36	0 04	0 23	34	0 18	0 20	32

*) One was an established female

B Experiments (total number 35) which had returned to normal activity (shortened from pre-exp average by less than 0 6 h)*

Period number	1	2	3	4	5
Number of experiments	17	24	26	29	30

*) One experiment had not returned to normal activity when it dropped out after circadian period no 3

change in the roostings after an EPD indicate that waking up and roosting are not controlled in the same manner by one basal clock. If they were controlled in the same manner then all properties of the normal rhythm should have been re-established after the first waking up (W_1) as there was no further change in the length of the circadian period ($W_1W = W_pW_p$ in Fig. 2) after it. This is clearly not the case (Table II A). A separate discussion of the effects on the waking up and the roosting by an EPD is thus warranted.

b. Analysis of the effects on the waking up time

The effect of an EPD on the waking up time the following morning is shown in Fig. 3. In experiments with EPD's longer than 3 hours there is a considerable variation in the obtained delay. A slight increase in the delay with increasing length of the EPD can be seen. This increase is more due to an increase in the maximal values than in the minimal ones. This minimal induced delay is fairly stable at approximately 1.5 hours with all the EPD's used. The maximal delay increases somewhat with increasing EPD's. In only one case, however, the delay corresponds to the EPD (3 hours). There may be an upper limit to the delays.

Figure 4 A and B show the relationship between the induced delay in the first waking up and the average length of 4 pre-experimental rest and circadian periods. The rest and circadian period immediately prior to the EPD have not been included in this average. Only experiments with EPD's of more than 3 hours are included. The length of the circadian period had no detectable influence. A long average pre-experimental rest period gives a short delay, a long average pre-experimental activity gives the opposite, a long delay. The corresponding regression and correlation coefficients are given in Table III. The delay of the first waking up showed a negative correlation with the four pre-experimental rest periods and a positive correlation with the corresponding activity. The correlation with rest is slightly stronger. Since activity and rest are correlated ($r = -0.97$) it was of interest to calculate the partial correlation between delay and activity and rest. With pre-experimental activity kept constant, the partial correlation coefficient between delay of first waking up and rest was -0.48 ($P < 0.01$) and with rest kept constant the partial correlation coefficient between delay of first waking up and activity was -0.32 ($P > 0.05$). The partial correlation coefficients thus point towards pre-

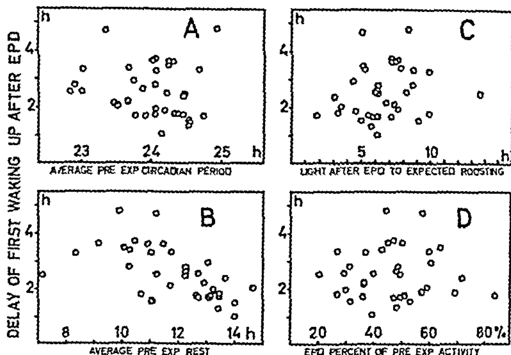


Fig 4 Influence of different variables on the delay obtained in the first waking up after an EPD (EW_1) Average pre experimental circadian period (A) and rest (B) have been calculated on 4 values The ones immediately prior to the EPD have been omitted Light after EPD up to expected roosting (C) corresponds to LR_p in Fig 2 All experiments with an EPD of more than 3 hours have been used (n=36) $\bar{x}$ was in A 24 01 h B 11 8[illegible] h C 6 57 h and D 46 6 %

experimental rest as the important variable for the correlation with delay in the first waking up

Fig 4 C and D show that the absolute duration of light from the end of the EPD to the time when the normal roosting should have occured (LR_p Fig 2) had no detectable influence on the delay of the first waking up Nor is the delay influenced by the relative amount of the pre experimental activity which was covered by the EPD The delay was approximately the same when much of the activity was covered as when only a small part was covered by the EPD

In Table III the regression and correlation coefficients which have just been discussed are shown The delay of the first waking up depended to a small degree on the length of the EPD but there was no correlation with the light time after the EPD to the expected roosting nor with the relative amount of the pre experimental activity covered by the EPD There has been no tendency to give longer EPDs

Table III Regression (b) and correlation (r) coefficients with delay of waking up as dependent variable. The experiments in Table II A have been used (I PD > 3 h). Average pre experimental activity, rest and circadian period have been calculated on 4 values (the one immediately prior to the I PD has been omitted) in the first 3 coefficients and on 5 values in the others. Letters inside brackets refer to Fig. 2. s_b and s_r see methods

X	Y	n	b Y on X	s_b	r	s_r	P
Average pre exp activity (W_pR_p)	Delay of first waking up after I PD (FW_1)	36	0 [illegible]9	0 08	0 54	0 14	< 0 001
Average pre exp rest (R_pW_p)	» » » » » » » »	36	−0 34	0 07	−0 62	0 13	< 0 001
Average pre exp circadian period (W_pW_p)	» » » » » » » »	36	−0 34	0 [illegible]8	−0 [illegible]1	0 17	> 0 05
Length of I PD (W_aI)	Delay of first waking up after I PD (IW_1)	36	0 [illegible]0	0 09	0 34	0 16	< 0 05
Light after EPD to expected roosting (LR_p)	» » » » » » » »	36	0 08	0 07	0 20	0 17	> 0 05
EPD as % of pre-exp activity	» » » » » » » »	36	0 00	0 01	0 03	0 17	> 0 05
Average pre exp rest ($\overline{R_pW_p}$)	Length of EPD (W L)	36	0 02	0 16	0 02	0 17	> 0 05

to birds with short rest periods and this is shown by the last coeffi cients in Table III

After the end of a short EPD there was a circadian period of pre experimental length to the next waking up ($LW_1 = \overline{W_pW_p}$ Fig 2) The clock controlling the waking up times seemed to be started by the light This was not the case with the long EPD s Here the clock must have started during the EPD The only possibility at present to get an approximate estimate of when the clock started is to assume that there was a normal length of the circadian period to the next waking up ($W_bW_1 = \overline{W_pW_p}$ Fig 2) in the same manner as with the short EPD s This assumption implies that the real delay of waking up does not occur at the end of the night following the EPD (W_1) but during the EPD proper The length of the rest in the circadian period prior to the EPD (the rest period extending to the hypothetical waking up W_b) then consists of two parts the time from the roosting to the attempted waking up (R_pW_a Fig 2) and the delay of the waking up (EW_1 Fig 2)

Fig 3 shows the length of these real rest periods The *observed*

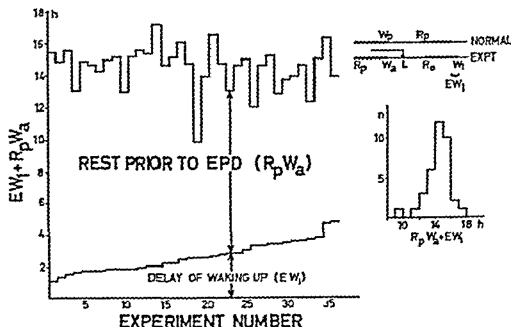


Fig 3 The sum of the rest period immediately prior to the EPD (R_pW_a) and the obtained delay in the first waking up after the EPD (EW_1) The delays are arranged according to their durations in the left part of the figure The distribution of the sums are shown in the right part Part of Fig 2 is inserted to show the variables used All experiments with an EPD of more than 3 hours have been used (n=36)

delays of the waking up times (EW_1) have been arranged according to their length. To these have been added the corresponding *observed* rest periods prior to the EPD (R_pW_1, Fig. 2). The sum has an approximately normal distribution with a mean of 14.5 h and a S.E. of 0.23 h (n=36). It is largely independent of the delay of waking up ($r=-0.18 \pm 0.17$). This is an indication that the last pre-experimental rest may determine the delay obtained by a long EPD. (The correlation between the rest period immediately prior to the EPD and the delay in the first waking up was −0.67.) If the rest is long the delay is small. If the assumption is correct that the sum is the real rest period, then during an EPD the clock regulating the waking up times tends to start a certain time after the previous roosting (14.5±0.23 hours) and the start could mark the end of the rest period (see discussion pp. 34–37).

c. *Analysis of the effects on the first roosting*

The effect of an EPD on the roostings was more gradual than the effect on the waking up. The delay obtained in the first roosting

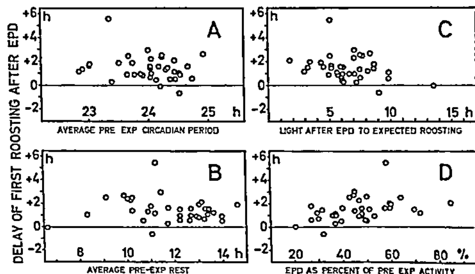


Fig. 6. The influence of different variables on the delay obtained in the first roosting after an EPD (FR). Average pre-experimental circadian period (A) and rest (B) have been calculated on 4 values. The ones immediately prior to the EPD have been omitted. Light after EPD up to expected roosting (C) corresponds to LR_p in Fig. 2. All experiments with an EPD of more than 3 hours have been used (n=36). $\bar{x}$ was in A 24.01 h, B 11.85 h, C 6.5[illegible] h and D [illegible] %.

after an EPD has been analysed in the same manner as the delay obtained in the first waking up to see if it was dependent on the same factors

In Fig 6 A and B the delay of the first roosting (ER_0 Fig 2) has been plotted against the average of 4 pre experimental rest and circadian periods The rest and circadian period immediately prior to the EPD have been omitted It is evident that neither the average length of the pre experimental circadian period nor the average pre experimental rest period had any detectable influence on the delay obtained in the first roosting The correlation and regression coefficients are given in Table IV They were not significantly different from zero The independance of the circadian period is the same as for the waking up times The pre experimental rest period had however an influence *on the delay of the waking up but not on the delay of the roostings* and thus indicates that the waking up and the roosting are not directly connected in the same manner to the same basal internal clock mechanism

Fig 6 C shows the delay of the first roosting plotted against the duration of light after the EPD up to the time when the birds should have roosted in a normal rhythm In Fig 6 D the delay is plotted against the length of the LPD as per cent of pre experimental activity This is comparable to Fig 4 C and D Fig 6 D shows that the delay of the first roosting increases with the part of the activity which is covered by the EPD Table IV shows the regression and correlation coefficients They are significant at the 0 01 level With duration of light to the expected roosting as the independent variable there is also a trend towards a larger delay with shorter light (Fig 6 C) but the corresponding correlation and regression coefficients are not significantly different from zero (Table IV) The correlation between the length of the LPD and the delay of the first roosting thus seems to depend more on the relative amount of activity covered by the EPD than on the absolute light time following the EPD up to the expected roosting Also in this regard the delay of the waking up times behave differently from the delay of the first roosting The delay of the waking up times were not influenced by the relative amount of activity which the LPD covered

d Relationship between delay of first roosting and delay of first waking up

An LPD always gave a delay of the first waking up (Fig 3) The

Table IV Regression (b) and correlation (r) coefficients with delay of first roosting after the FPD as dependent variable The experiments in Table II A have been used (FLD > 3 h) Average pre experimental activity rest and circadian period have been calculated on 4 values (the one immediately prior to the LPD has been omitted) in the first three coefficients and on 5 values in the others Letters inside brackets refer to Fig 2 s_b and s_r see methods

X	Y	n	b Y on X	s_b	r	s_r	P
Average pre exp activity (W_pR_p)	Delay of first roosting after LPD (LR_O)	36	0 05	0 10	0 08	0 17	> 0 05
Average pre exp rest (R_pW_p)	» » » » » » »	36	−0 05	0 11	−0 07	0 17	> 0 05
Average pre-exp circadian period (W_pW_p)	» » » » » » »	36	−0 02	0 32	−0 01	0 17	> 0 05
Length of LPD (W L)	Delay of first roosting after FPD (FR_O)	36	0 33	0 10	0 50	0 15	< 0 01
Light after LPD to expected roosting (LR_p)	» » » » » » »	36	−0 15	0 08	−0 32	0 16	> 0 05
LLD as % of pre exp activity	» » » » » » »	36	0 03	0 01	0 46	0 15	< 0 01

roostings as a rule (with two exceptions see for instance Fig 6) were also delayed This means that in these experiments there was a positive correlation between the effect on the waking up (EW_1) and the effect on the first roosting (ER_0) after an EPD ($r = +0.71$)

In the normal rhythm the roostings usually are much more variable than the waking up times An increase in activity by delayed roosting nearly always leads to a decrease in rest This is shown by the filled circles in Fig 7 A These controls have been obtained by assuming that there was an EPD of 0 hours in the circadian period immediately prior to the real experiment (the material consists of the series with real EPDs longer than 3 hours) The changes seen in the last period prior to the EPD have been calculated from the average of the four preceding periods The filled circles in Fig 7 A show that in the normal rhythm there is a strong negative correlation ($r = -0.94$) between delay of first roosting (increase in activity) and increase in the following rest period

The corresponding changes obtained after an EPD of more than 3 hours are plotted as open circles in Fig 7 A These changes have been calculated from the average of the 5 circadian periods prior to the EPD It is evident from Fig 2 that the difference between the delay in the

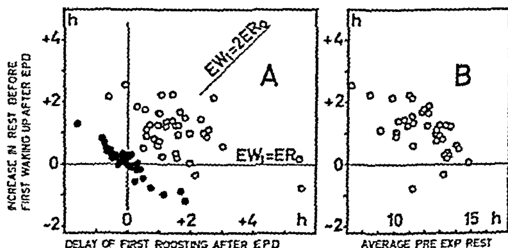


Fig Influence of some variables on the increase in the first rest period afte the EPD orresponds to $EW_1 - ER_0$ Fig 2) Open circles are experiments with an EPD f more than 3 hours (n=3?) Filled circles in A (derived from the same experiment illustrate the situation in the circadian period immediately prior to th ea EPD with an EPD of 0 hours In these experiments the delay (x=—0.01 h) ar 5 increa e x=0.00 h) are against the average of the 4 immediately preceding period s was in B 11.65 h

first waking up and the delay in the first roosting (EW_1—ER_0) is equal to the increase in the first rest period after the EPD (R_0W_1—R_pW_p) The open circles in Fig 7 A show that the usual negative correlation was reduced after an EPD of more than 3 hours (r=—0 52 as compared with r=—0 94) The delay in the first roosting was not a fixed part of the delay in the following waking up

In Fig 7 B the increase in the rest period (EW_1—ER_0) has been plotted against pre experimental rest the average rest in the five circadian periods prior to the EPD The experiments with an EPD of more than 3 hours have been used There was a negative correlation (r=—0 60) of the same magnitude as between pre experimental rest and delay of first waking up (r=—0 62 see p 17 and Table III) The negative correlation between pre experimental rest and delay of first waking up —0 62 could thus conceivably be explained by the other correlation —0 60 and be due to the changes occurring in the length of the first rest period after the EPD However as will be shown below the negative correlation between delay of first roosting and increase in the rest period after the EPD disappeared under certain conditions while the negative correlation between delay of first waking up and pre experimental rest was still evident (chapter II part 2) Thus the causal relation probably works in the other direction and the interpretation of the correlation given on p 21 is not invalidated by the present results This will be further discussed below

2 Influence of a short light period before the enforced period of darkness

In these experiments a short period of light was given to the birds when they left the night perch by an automatic clock device (light periods longer than 1 min were delivered manually) After this light time the light in the cage was extinguished and an enforced period of darkness (LPD) followed in the same manner as in the previous experiments Except with very short light times the birds always immediately left the night perch The birds were thus sitting either on the day perch or on the floor during the following EPD Some experiments with 1—2 5 and 60—600 sec of light prior to an EPD of 1 hour have been published earlier (Wahlstrom 1965)

Tables V A and B summarize the results of the present experiments but do not include the previous ones The changes occurring in cir

Table I Changes in activity rest and circadian period after an EPD of 1 and 4 h preceded by short light Changes calculated as increase over the average of the five circadian periods prior to the EPD

I EPD 1 hour

Variable	Series	Pre exp av h	±SE	Number of birds	n	Light range sec	Duration of EPD h	(range)	Increase over pre exp average in period no 0 h	0 ±SE	1 h	1 ±SE	2 h	2 ±SE
Circadian period	D_1^b	23 85	0 13	6[a]	10	0	1 04	(0 98—1 23)	0 96	0 09	0 02	0 09	0 20	0 07
	$L_{<1}D_1$	24 01	0 14	5	6	< 1	1 00	(0 78—1 17)	0 86	0 07	—0 02	0 09	—0 07	0 06
	$L_{15}D_1$	23 88	0 25	6	13	15	0 96	(0 89—1 10)	0 57	0 09	0 08	0 11	0 02	0 09
	$L_{60}D_1$	23 94	0 15	9[a]	11	60—120[c]	0 97	(0 89—1 03)	0 11	0 07	0 00	0 10	—0 06	0 08
Activity	D_1^b	11 52	0 76	6[a]	10	0	1 04	(0 98—1 23)	0 33	0 14	—0 29	0 13	—0 05	0 12
	$L_{<1}D_1$	13 18	0 96	5	6	< 1	1 00	(0 78—1 17)	0 29	0 33	—0 20	0 29	—0 50	0 17
	$L_{15}D_1$	13 27	0 77	6	13	15	0 96	(0 89—1 10)	0 40	0 33	—0 46	0 41	—0 05	0 51
	$L_{60}D_1$	12 64	0 51	9[a]	11	60—120[c]	0 97	(0 89—1 03)	0 22	0 21	0 04	0 25	—0 16	0 17
Rest	D_1^b	12 33	0 76	6[a]	10	0	1 04	(0 98—1 23)	0 63	0 14	0 30	0 16	0 25	0 15
	$L_{<1}D_1$	10 82	0 96	5	6	< 1	1 00	(0 78—1 17)	0 57	0 27	0 18	0 23	0 43	0 14
	$L_{15}D_1$	10 61	0 56	6	13	15	0 96	(0 89—1 10)	0 17	0 36	0 54	0 35	0 07	0 16
	$L_{60}D_1$	11 30	0 12	9[a]	11	60—120[c]	0 97	(0 89—1 03)	—0 12	0 19	—0 04	0 18	0 09	0 13

a) One bird in the series was an established female b) Identical with D_1 Table I c) One experiment performed with 120 sec 10 with 60 sec

Table V (continued)

B FPD 4 hours

Variable	Serie	Pre exp av h	±SE	Number of birds	n	Light range sec	Duration of FPD h	(range)	Increase over pre exp average in period no 0 h	±SE	1 h	±SE	2 h	±SE
Circadian period	$D_4{}^d$	23.72	0.17	7	8	0	4.02	(3.67–4.27)	1.99	0.20	−0.14	0.08	−0.16	0.06
	$L_{60}D_4$	23.72	0.19	8	13	60–120[e]	3.89	(3.71–4.15)	1.12	0.20	0.09	0.10	0.05	0.08
	$L_{300}D_4$	23.69	0.16	7	7	300	3.93	(3.76–4.03)	0.91	0.29	0.26	0.13	0.02	0.10
	$L_{600}D_4$	23.68	0.10	7	10	600–780[f]	4.07	(4.00–4.20)	0.75	0.19	0.06	0.10	0.05	0.10
	$L_{1800}D_4$	23.61	0.21	9	9	1800	4.02	(3.89–4.28)	0.56	0.16	0.18	0.17	0.21	0.10
Activity	$D_4{}^d$	11.64	0.50	7	8	0	4.02	(3.67–4.27)	0.72	0.24	−0.78	0.20	−0.35	0.31
	$L_{60}D_4$	11.88	0.48	8	13	60–120[e]	3.89	(3.71–4.15)	0.67	0.51	0.19	0.29	−0.14	0.30
	$L_{300}D_4$	12.41	0.66	7	7	300	3.93	(3.76–4.03)	0.93	0.36	0.30	0.26	0.27	0.23
	$L_{600}D_4$	12.75	0.46	7	10	600–780[f]	4.07	(4.00–4.20)	0.97	0.26	0.20	0.32	0.24	0.36
	$L_{1800}D_4$	12.27	0.68	9	9	1800	4.02	(3.89–4.28)	1.48	0.59	0.56	0.61	0.21	0.51
Rest	$D_4{}^d$	12.07	0.45	7	8	0	4.02	(3.67–4.27)	1.26	0.19	0.64	0.16	0.19	0.30
	$L_{60}D_4$	11.84	[illegible]	8	13	60–120	3.89	(3.71–4.15)	0.45	0.41	−0.10	0.24	0.20	0.26
	$L_{300}D_4$	11.27	0.71	7	7	300	3.93	(3.76–4.03)	−0.03	[illegible]	−0.04	0.20	−0.25	0.20
	$L_{600}D_4$	10.93	0.44	7	10	600–780[f]	4.07	(4.00–4.20)	−0.23	0.30	−0.14	0.25	−0.19	0.29
	$L_{1800}D_4$	11.34	0.57	9	9	1800	4.02	(3.89–4.28)	−0.92	0.58	−0.38	0.50	0.00	0.45

d) Is one part of the experiments in Table II e) 4 exp with 120 sec 9 with 60 sec f) 1 exp with 780 sec 9 with 600 sec.

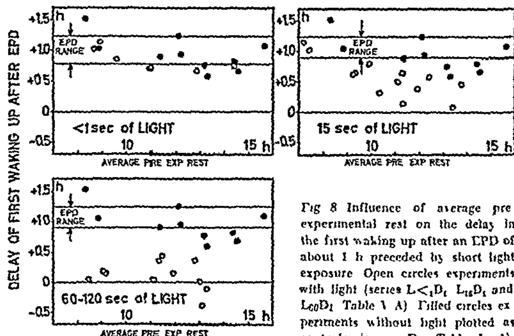


Fig 8 Influence of average pre experimental rest on the delay in the first waking up after an EPD of about 1 h preceded by short light exposure Open circles experiments with light (series $L_{<1}D_1$ $L_{15}D_1$ and $L_{60}D_1$ Table V A) Filled circles experiments without light plotted as controls (series D_1 Table V A) Average pre experimental rest calculated on 4 values The ones immediately prior to the EPD have been omitted $\bar{x}$ in the series with no light was 12.38 h <1 sec of light 10.00 h 15 sec of light 10.66 h and 60—120 sec of light 11.34 h

circadian period no 0 will be discussed below with the aid of figures The changes in the circadian periods following no 0 will be dealt with first

The length of the circadian period had returned to the pre experimental length in period no 1 except in series $L_{300}D_4$ The activity was decreased and the rest increased by earlier roosting in circadian period no 1 in series D_1 and D_4 (no light interposed) Similar changes are seen in experiments with very short light periods prior to an EPD of 1 hour (series $L_{<1}D_1$ and $L_{15}D_1$) but not in the experiments with 60 sec or more of light prior to an EPD of 4 hours (series $L_{60}D_4$ $L_{300}D_4$ $L_{600}D_4$ and $L_{1800}D_4$) The gradual changes in the roostings seen after EPD s without light (discussed in chapter II part 1 a) thus disappear when 60 sec of light or more is interposed before the EPD

Fig 8 shows the delay of the first waking up (seen as an increase in the length of the circadian period during period no 0 in Table V A) plotted against the average of four pre experimental rest periods (the rest immediately prior to the EPD has been omitted) for series D_1 $L_{<1}D_1$ $L_{15}D_1$ and $L_{60}D_1$ of Table V A Series D_1 is plotted as a control (filled circles) in all parts of Fig 8 It is seen that 60—120 sec

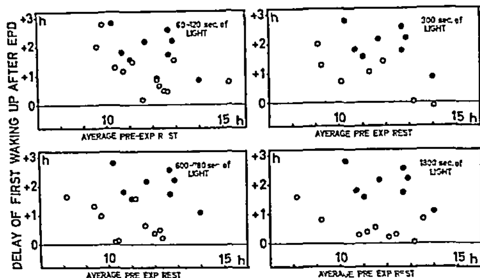


Fig 9 Influence of average pre experimental rest on the delay in the first waking up after an EPD of about 4 hours preceded by short light exposure Open circles experiments with light (series $L_{60}D_4$ $L_{300}D_4$ $L_{600}D_4$ and $L_{1800}D_4$ Table V B) Filled circles experiments without light plotted as controls (series D_4 Table V B) Average pre experimental rest was calculated on 4 values The ones immediately prior to the EPD have been omitted $\bar{x}$ in the series with no light was 12 00 h 60—120 sec of light 11 81 h 300 sec of light 11 [illegible]7 h 600—780 sec of light 10 [illegible]5 h and 1800 sec of light 11 33 h

of light almost totally inhibited the effect of the EPD waking up was not delayed 15 sec of lights was more inhibiting than <1 sec of light although even this flash of light may have had a slight effect the delay in series D_1 was 0 96 as against 0 86 h in series $L_{<1}D_1$ Furthermore the inhibiting effect of 15 sec of light decreased with increasing average pre experimental rest In fact after very short rest periods 15 sec of light had no inhibiting effect at all on the delay induced by an EPD of one hour

In Fig 9 the delay of the first waking up is plotted against the average of four pre experimental rest periods in the same manner as in Fig 8 for the experiments in series D_4 $L_{60}D_4$ $L_{600}D_4$ and $L_{1800}D_4$ (Table V B) Series D_4 is plotted as a control in all parts of Fig 9 The figure shows that in all series the delay obtained was dependent on the pre experimental rest The light prior to the EPD diminished the induced delay over the whole range of durations of pre experimental rest 1800 sec of light was clearly more inhibiting than 60—120 sec The results of the experiments with 300 and 600—780 sec of light were

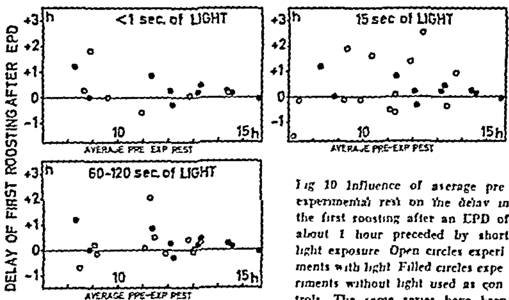


Fig 10 Influence of average pre experimental rest on the delay in the first roosting after an EPD of about 1 hour preceded by short light exposure Open circles experiments with light Filled circles experiments without light used as controls The same series have been used as in Fig 8 Average pre experimental rest was calculated on 4 values The ones immediately prior to the EPD have been omitted

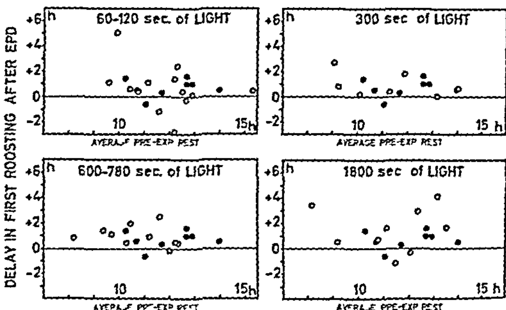


Fig 11 *Influence of average pre-experimental rest on the delay in the first roosting after an EPD of about 4 hours perceded by short light exposure Open circles experiments with light Filled circles experiments without light used as controls. The same series have been used as in Fig 9 Average pre experimental rest was calculated on 4 values The ones immediately prior to the EPD have been omitted.*

intermediate After 1800 sec of light the EPD seemed to have an effect only in experiments with short pre experimental rest periods If pre experimental activity was used instead of rest the opposite relation was obtained Similarly to the results with EPD without light (Fig 4) there was no relation between pre experimental circadian period and the delay of the first waking up

A comparison between Fig 8 and 9 reveals that 60—120 sec of light could almost completely prevent the effect of an EPD of one hour but only diminish that of an EPD of 4 hours

It is seen in Fig 9 that the points of D_4 and $L_{60}D_4$ cluster around lines of roughly similar slope The regression coefficients calculated on admittedly few experiments were —0 18 and —0 28 respectively A very rough estimate of the average effect of 60—120 sec of light prior to an EPD of 4 hours has been obtained by calculating the vertical distance between the two regression lines at an average pre experimental rest period of 12 07 h which is the mean of series D_4 This distance was 0 93 h In the experiments with an EPD of 1 hour (Fig 8) the corresponding distance was 0 85 h It thus seems as if 60—120 sec of light reduced the effect of an EPD on the delay of the waking up with approximately the same amount irrespective of the duration of the following EPD

In Fig 10 and 11 the corresponding delays in the first roosting in the series shown in Fig 8 and 9 have been plotted against the average of four pre experimental rest periods (the one immediately prior to the EPD has been omitted) In contrast to the findings regarding the first waking up no clearcut relationship to the pre experimental rest was obtained (a slight negative correlation can perhaps be seen in series $L_{600}D_4$ Fig 11) Another contrast is that the delay in the first roosting is not markedly reduced by light prior to the EPD Note the difference in scale of the ordinates between Fig 8 9 and 10 11

The delay in the first roosting was not reduced by increased duration of light prior to the EPD in the present experiments but there was a gradual reduction in the delay of the first waking up A consequence of this is that the first rest period after the EPD was gradually reduced with longer light times In series $L_{600}D_4$ and $L_{1800}D_4$ there was even a decrease in the first rest period after the EPD below the pre experimental average (Table V B)

The difference between the first rest period after an EPD of 4 hours preceded by light and the average of the five pre experimental rest periods (equal to EW_1—ER_0) has been plotted against delay of the

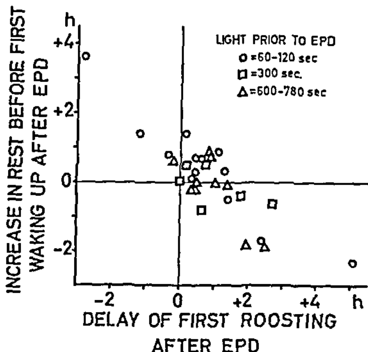


Fig 12 Influence of the delay in the first roosting after an EPD of about 4 hours preceded by short light exposure on the following rest period Some of the series with light in table V B have been used

first roosting after the EPD and is shown in Fig 12 Compared with the *open circles in Fig 7 A (EPD without light) it shows a much* stronger negative correlation As the delay of the first roosting was approximately the same as with no light prior to the EPD and the delay of the first waking up was reduced by light there are more points in the lower right quadrant and the conditions approach those seen without an LPD (filled circles in Fig 7 A) Several experiments thus showed a decrease in the first rest after an LPD The observed delay in the first waking up in the experiments with light prior to an EPD of 4 hours no longer usually consists of two positive parts as in the experiments without light namely a delay in the first roosting and an increase in the first rest period after the EPD

In Fig 13 the changes in the first rest period after the EPD in series D_4 $L_{60}D_4$ $L_{300}D_4$ $L_{600}D_4$ and $L_{1600}D_4$ have been plotted against average rest in the five circadian periods prior to the EPD The negative correlation ($r = -0.60$) found in Fig 7 B for EPDs of more than 3 hours without light is still evident in the smaller number of experiments

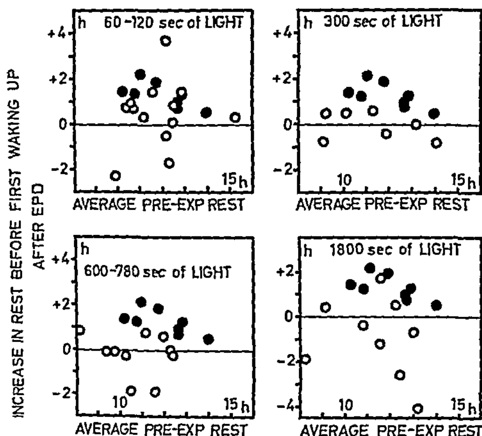


Fig 13 Influence of average pre experimental rest on the increase in the first rest period after an EPD of about 4 hours preceded by short light exposure The series in table V B have been used Open circles experiments with light Filled circles experiments without light plotted as controls

with EPDs around 4 hours (D_4 filled circles in all parts of Fig 13) There is however no such correlation in the experiments with light prior to the EPD

The observed negative correlation between delay of first waking up and pre experimental rest thus does not seem to be due to changes in the first rest period after the EPD as the correlation between increase in first rest period and pre experimental rest was lost in the experiments with light prior to an EPD of four hours This indicates that the changes in the clock which are measured as a delay of the first waking up occur already during the EPD

3 Discussion

The different kinds of experiments performed with EPD s have shown that there are several differences between the control of waking up and of roosting in the self selected circadian rhythm in the canary Table VI summarized the results The delay in the waking up times after an EPD covering the start of the activity is fully shown by the first waking up following the EPD and is dependent on the pre experimental rest *The delay induced in the roostings appears more gradually* in 2—4 circadian periods after the experiment depending on the length of the EPD The delay obtained in the first roosting after the EPD is not dependent on the pre experimental length of the rest period There is, however, a correlation between the delay in the first roosting and the relative amount of activity covered by the EPD No such correlation exists with regard to the delay in the waking up Furthermore the ability of a short period of light before the EPD to inhibit the delay obtained by it in the first waking up is dependent on the pre experimental rest but no such dependence could be established for the corresponding roosting The delay of the first roosting after the EPD was in fact not appreciably diminished by the short light times used prior to the EPD It has earlier been shown that in birds with divided activity

Table VI Summary of differences between roosting and waking up The data for undisturbed rhythm are from Wahlström (1961 b)

Experiments with undisturbed rhythm		Roosting	Waking up
		Large variability	Small variability
		The effect of the EPD on the delay of*	
		Roosting	Waking up
Experiments with enforced periods of darkness (EPD s)	comes gradually	Yes	No
	depends on pre exp rest	No	Yes
	depends on activity covered by EPD	Yes	No
	is inhibited by short light	No	Yes

*) Delay of roosting = increase in activity from pre exp average
Delay of waking up - increase in circadian period from pre exp average

(a rest interposed in the activity) an earlier roosting than expected does not influence the following waking up (Wahlström 1964 b). In the normal rhythm it is also much more common to find a large variation in the time of roosting compared with the time of waking up (Wahlström 1964 b). There are thus several indications that the canary has two partially independent mechanisms which regulate the self selected rhythm. One presumable is a steady clock mechanism which controls the waking up times. The other mechanism controls the amount of activity and rest within the circadian period.

The clock mechanism controlling the waking up times seems to be started on its new cycle by light in some of the present experiments and this is probably what happens in the normal rhythm. After a short EPD (less than 1.5 h) the delay of the first waking up corresponds to the length of the EPD. There is a normal circadian period from the end of the EPD to the next waking up. If one minute of light is allowed before an EPD of one hour then the EPD has virtually no effect on the next waking up, the delay is inhibited by light and there is a normal circadian period from the short light exposure to the next waking up.

Also during the long EPD's it is unlikely that the clock starts on its new cycle at the attempted waking up (when the bird is unsuccessful in obtaining light). This does not seem to be the case in the experiments with short EPD's and the bird has no way of knowing if it is going to be a long or a short EPD. If it starts at the end of a long EPD there ought to be a normal circadian period from the end of the EPD to the next waking up, as in the experiments with short EPD's. This not being the case the conclusion must be that light is not necessary to start the clock controlling the waking up times.

In the experiments with short EPD's the clock controlling the waking up times seems to start at the end of the EPD. The EPD is thus a prolongation of the previous circadian period. Since the first part of a long EPD is a short one at least part of the measured delay in the first waking up must be due to a prolongation of the circadian period prior to the EPD. If it is assumed that there is a normal circadian period from the start of the clock to the first waking up after the EPD then the whole measured delay in the first waking up is an increase in the circadian period prior to the EPD. In fact this is the only possibility at present to get an approximate estimate of the time when the clock controlling the waking up times starts during a long EPD (assuming that the clock runs at constant speed once it has started)

If the delay in the first waking up induced by the EPD is added to the rest period belonging to the circadian period prior to the EPD (Fig. 5) the sums fluctuate around 14.5 hours and is not correlated with the delay. This indicates that the clock mechanism controlling the waking up is not totally independent of the mechanism controlling the roosting. The clock seems to start a certain time (14.5 ± 0.23 h) after the previous roosting if no light occurs at the attempted waking up. If this is true then one question of importance immediately rises. Can the bird be awake without the clock starting? The attempted waking up — the first hops on the night perch — could mean that the birds really woke up for good and that afterwards they did not fall asleep again. If this is the case, then during an EPD the start of the clock is dissociated from the waking up, and light must have a direct influence on the clock mechanism (and not only by waking the bird). If the birds did go to sleep again after the first hop which failed to produce light, then the attempted waking up really is only an *attempted* waking up, and the clock could be started by a certain degree of alertness of the bird. Light could then influence the clock not directly but through a change in wakefulness.

The sporadic movements on the perches recorded in some experiments with EPDs cannot be used as evidence in favour of either of these alternatives. The fairly constant time relationship between the roosting prior to the EPD and the assumed clock start during long EPDs indicates that clock start and wakefulness may be related. Even if there is no light in the cage the birds are unable to sleep for more than a certain time and when they wake up the clock starts. A short light exposure prior to the EPD wakes up the birds and starts the clock more easily if they have rested for a long time.

The picture is, however, complicated by the effect of 1—2 min of light prior to an EPD of 1 and 4 hours. 1—2 min of light inhibits the effect of an EPD of 1 hour. The clock starts at the time of the short light. With the same duration of light but an EPD of 4 hours there is a delay in the first waking up, although the clock presumably must have run at least for the first hour of the EPD. The delay was diminished by approximately the same amount (0.9 hours) by 1—2 min of light whether the EPD was 1 or 4 hours. Thus 1—2 min of light seem to make the clock run for about 0.9 hours before it stops again. It could be that this is the time it takes for the bird to go to sleep again after the short period of light. If the light period is much

longer the clock seems to be started for good This is seen in birds with divided activity (Wahlstrom 1964 b)

Further complications arise from the results with drugs With barbiturates (Chapter IV) it is possible to induce a small but measurable delay of the waking up This delay however does not act as an EPD The barbiturate induced delay is compensated and the following waking up occurs at the time expected from the pre experimental periods

At present no definite conclusions regarding the relationship between the clock start and the state of wakefulness of the canaries during experiments with EPD s can be reached In the normal rhythm they seem to be connected but this could be a coincidence of light and waking up occurring together This question is however accessible to further experiments and these are in progress

It is possible to induce a delay in the start of locomotor activity by various manipulations with light and darkness in rhythms which are not self selected A review of the work in this field has been given by Aschoff (1963) Only some recent examples will be mentioned here

If the locomotor activity rhythm of the chaffinch (*Fringilla coelebs*) is regulated by light and darkness then a phase shift of the Zeitgeber (light darkness) by prolongation of the darkness induces a delay in the start of activity (Aschoff and Wever 1963) In nocturnal animals kept in constant darkness a short light (which ought to be equivalent to darkness in a light active animal) given within a few hours after the start of locomotor activity induces a delay in the following activity start This has been shown for the flying squirrel (*Glaucomys volans*) and the hamster (*Mesocricetus auratus*) by DeCoursey (1961 and 1964)

Chaffinches in constant darkness have a longer rest and a longer circadian period calculated from the start of locomotor activity than chaffinches in various intensities of constant light (Aschoff et al 1962) The rest and the circadian period follow the general rules formulated by Aschoff (1959 and 1960) The connection between these experiments in the chaffinch and the results with long EPD s in the canary cannot be established at present

CHAPTER III

EFFECTS OF MONOAMINE OXIDASE INHIBITORS ON THE CIRCADIAN RHYTHM

1 Results with phenprazine

Phenprazine has been given orally in different doses With doses smaller than 60 mg/kg no constant effects were obtained on the rhythm and these experiments have not been used The other experiments performed with phenprazine have been divided into two series One series with less than 100 mg/kg consists of 13 experiments with 60 mg/kg 5 with 75 mg/kg and 3 with 85 mg/kg Five birds were used (one was an established female) One series with 100 mg/kg or more consists of 9 experiments with 100 mg/kg 12 with 125 mg/kg 3 with 150 mg/kg and 6 with 175 mg/kg Four (including the female) of the 5 birds which received the low dose were used Two further experiments were excluded from these series due to technical reasons There were no casualties due to the drug

The behaviour of the birds was very little influenced after these doses of phenprazine

The results are shown in Fig 14 The effect on the rhythm has been calculated for each experiment as the difference from the pre experimental period consisting of the 5 circadian periods immediately prior to the drug administration these differences have been averaged The drug was given during circadian period no 0

Fig 14 (AM) shows that phenprazine given early in the activity had an influence on the length of the activity and rest The activity was decreased during the first 4 circadian periods after the drug (phase I) Later there was an increase in activity which declined gradually over approximately 10 periods (phase II) The changes in the rest were the mirror image of the changes in the activity since circadian period was almost unchanged A tendency towards a longer circadian period immediately after the higher dose of the drug and then a shorter one for 3—4 more periods can be seen but the deviations are small

With the exception of period no 0 a higher dose gave a more pronounced effect

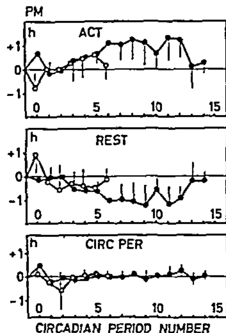


Fig 14 Effect of a single oral dose of pheniprazine on the circadian rhythm Increases have been calculated over the average (shown below) of the 5 periods immediately prior to the drug administration in period no 0 Experiments with 100—175 mg/kg marked ● and with 60—85 mg/kg O Vertical bars (sometimes only given for every second point and plotted in only one direction) 2 × S E In the N experiments S E was for rest and circadian period 2 91 and 3 01 in period no 0 and 4 18 and 3 90 in period no 1 respectively Adm time is the average time from waking up to drug administration

	AM O	AM ●	PM O	PM ●	N ●
Act h	11.27	10 46	10 44	9 89	11 02
S E	0 49	0 40	0 89	0 72	0 61
Circ per h	23 94	23 74	23 92	23 66	23 86
S E	0 17	0 17	0.34	0 27	0 31
Adm time h	2.36	2 06	8 1[illegible]	7 [illegible]	14 13
n	12	16	9	9	5

In Fig 14 (PM) the corresponding changes in the circadian rhythm are shown when phemprazine was administered late in the activity period With few exceptions the pattern of the changes were the same as in the AM experiments Instead of a decrease in activity in circadian period no 0 there was an increase with the higher dose The increase in activity and corresponding decrease in rest (phase II) which with the AM dose occurred after circadian period 5 appeared earlier here and was more pronounced The slight changes in the circadian period were the same as in the AM experiments On phase II the higher dose gave a more pronounced effect

The changes obtained after drug administration during the rest period are shown in Fig 14 (N) In all the experiments the birds were if possible handled and given distilled water through the stomach tube in the periods before and after the drug administration and in the experiments seen in Fig 14 (N) this was done during the rest to exclude any possible effects of handling on the rhythm In the N experiments the changes in activity and rest (phase I) were more pronounced during the first 5 circadian periods than in the correspon ding AM and PM experiments The standard errors were also much larger (note the difference in scale of the ordinate) There was how ever an increase in activity during period no 0 which is similar to the change after the large doses in the PM series The increase of activity (phase II) after period 5 is of approximately the same order as after an AM administration The changes in the first two circadian periods and the very large standard error obtained were mainly due to two experiments in one the circadian period was first very short and then very long and in the second the long circadian period appeared first

In all experiments where phemprazine was given during activity the activity in period no 0 was decreased except when the high dose was given *late* in the activity (PM) In this last case the effect was the same as after a high dose given during rest The effect seen in the PM experiment depended on the time when the drug was given This is shown in Fig 15 where the change in activity during period no 0 is plotted against the time left until the birds should have roosted if the rhythm had been uninfluenced The small doses nearly always gave a decrease in activity With the high doses there were an increase in activity which was most marked when the drug was given close to the time when the birds were expected to roost If this time was in creased to 4—5 hours the activity was reduced as in the experiments

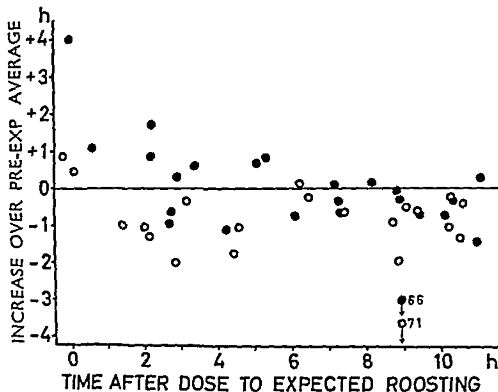


Fig 1a Influence of the time left after pheniprazine administration to the expected roosting on the changes obtained in that roosting All experiments with pheniprazine given during the activity are included Experiments with doses of 100—175 mg/kg are marked ● and experiments with 60—85 mg/kg O The changes in the roostings are calculated as increase in activity over the pre experimental average

with a small dose Thus there was a direct stimulating effect of the drug which lasted for perhaps 4 hours

In Table VII are shown the cumulated changes in the length of the circadian periods for the AM and PM experiments These cumulated changes show how much the waking up times have deviated from the time expected from the pre experimental periods when the rhythm had returned to normal These cumulated changes did not show any general trend Three were positive and one was negative The total sum of all of them was virtually zero Thus the increase obtained in the length of the first circadian period is compensated for in the following ones and on an average the waking up times return to the clock time expected from the pre experimental series of values

The effects of pheniprazine appeared to have a very long duration

Table III Cumulated changes (from the pre-experimental average) in length of circadian period after pheniprazine administration during activity

Dose mg/kg	Administered	Number of periods added	Number of experiments	Cumulated changes h
60—85	PM	7	9	−0.69
100—175	PM	15	9	+0.27
60—85	AM	7	12	+0.20
100—175	AM	15	16	+0.18
			Total	−0.04

To test if these long effects were really due to the drug or to some other factor a few AM experiments were performed in which pheniprazine 60—70 mg/kg was given twice with an interval of one week between the doses The second dose had a more pronounced phase II than the first dose indicating an overlap between the effect of the doses

2 Results with pargyline

Two different doses have been used Fifteen of the total of 18 experiments which could be evaluated have been performed with a dose of 350 mg/kg The other dose was 400 mg/kg and it was always given late in the activity The experiments have been performed in 4 birds One of these died after a dose of 350 mg/kg Another bird died after a dose of 400 mg/kg Two experiments with a dose of 350 mg/kg could not be evaluated as there was no apparent rhythm for a week after the drug

All the experiments were performed during the activity of the bird In all experiments the locomotor activity on the day perch was reduced after the drug administration Direct observations usually revealed a bird which was much calmer than usual The ability to use the perches was never decreased During the time equivalent to the first two circadian periods after the drug (approximately 48 hours) the birds usually sat inactive on either the day perch or the night perch There was thus either a long period of light in the cage after the drug (10 experiments) or a long period of darkness (4 experiments) In the experiments with the light on it could never be established if the bird was really asleep or not They did not take the sleeping position with

the bill under a wing and when the door was opened they were usually fully alert In four experiments there was the normal pattern with two activity periods and two rest periods during the first two circadian periods after the drug In these four experiments the rest was increased compared with the pre experimental rest

Due to the experiments with long light or dark periods no values for activity and rest during the first two circadian periods after the drug were obtained and these points have been omitted in Fig 16 An average length of the circadian period for the first two periods was

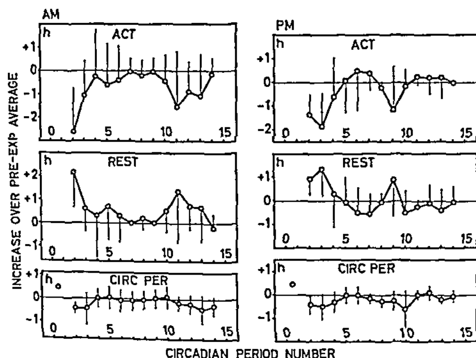


Fig 16 Effect of a single oral dose of pargyline on the circadian rhythm Increases have been calculated over the average (shown below) of the 5 periods immediately prior to the drug administration in period no 0 The AM dose was 350 mg/kg and the PM dose 350—400 mg/kg Vertical bars (sometimes plotted in only one direction) 2×S E

	AM	PM
Activity hours	12 96	14 33
S E	0 97	0 9
Circadian period hours	24 28	24 54
S E	0 25	0 2[illegible]
Average time from waking up to drug administration hours	2 39	9 49
n	8	10

obtained by dividing by 2 the time from waking up in period no 0 to the time of the waking up when the regular rhythm reappeared

The results obtained (shown as changes from the 5 pre experimental circadian periods) when pargyline was administered early in the activity are shown in Fig 16 (AM) The activity was decreased and the rest increased during the first periods which could be followed This corresponds to phase I after pheniprazine There was, however no clear phase II after pargyline given as an AM dose If the zero line is disregarded the different curves are remarkably similar to those obtained with pheniprazine (Fig 14) The standard errors were however larger in the pargyline experiments The slight changes in the circadian period were also similar The cumulated changes were —0 04 h after circadian period no 5 and —1 35 h after period no 14

The results obtained with PM administration are shown in Fig 16 (PM) The first decrease in activity and increase in rest (phase I) was of approximately the same magnitude as in the AM experiments After phase I there was a slight increase in activity and decrease in rest (phase II) in the PM experiments while there was no phase II at all in the AM experiments (Fig 16 AM) Phase II was more irregular, but with approximately the same maximal value as after pheniprazine given as an AM dose (Fig 14 AM) The similarity between the curves obtained with pheniprazine and pargyline is evident

The changes in the circadian period were slight but similar to those obtained with pheniprazine given as a PM dose The cumulated changes of the circadian periods were —0 33 h after period no 5 and —1 74 h after period no 14

3 Results with nialamide

Nialamide has been given in a dose of 1000 mg/kg 2 birds have been used but one died after the first drug administration

The single bird in which all the experiments were performed showed an unusual long time instability in the length of the circadian period Thus the results obtained must be taken with some reservation All experiments (3 AM and 3 PM) have been evaluated

The movements on the day perch were reduced after the drug administration This was more evident after the PM administration Direct observations of the bird did not reveal anything of importance The bird usually appeared normal and the ability to use the perches was uninfluenced except in one experiment where there was a slight incapacity for one hour after the drug No long light or dark periods similar to those obtained with pargyline were seen

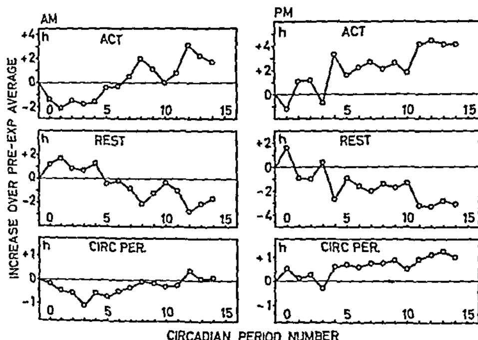


Fig 17 Effect of a single oral dose of nialamide on the circadian rhythm Increases have been calculated over the average (shown below) of the 5 periods immediately prior to the drug administration in period no 0 The dose of nialamide was 1000 mg/kg

	AM	PM
Activity hours	10 56	11 61
Circadian period hours	23 77	23 69
Average time from waking up to drug administration, hours	4 01	7 43
n	3	3

The effects of nialamide administered early in the activity are shown in Fig 17 (AM) The activity and rest showed the same pattern as after pheniprazine (Fig 14) with a marked phase I (decreased activity and increased rest) and a following phase II (increased activity and decreased rest) Phase I lasted for approximately 6 circadian periods The length of the circadian periods were decreased Compared to the results obtained with pheniprazine and pargyline this decrease was rather marked and lasted for a long time

In the PM experiments the activity was likewise decreased initially and the rest increased (phase I) This phase was much shorter than with AM administration Phase II (increase in activity) was very marked The pre experimental level of activity and rest had not returned in period no 14 The changes in the circadian period were also more prominent than those obtained with pheniprazine and pargyline In this case the circadian

periods were lengthened and they did not return to the pre-experimental value in those shown in the figure.

4. Discussion

There is no known drug which only inhibits the enzyme monoamine oxidase (MAO) and has no other effects. Many of the MAO inhibitors have several other pharmacological effects (Pletscher, Gey and Zeller, 1960) although nialamide and pargyline appear to be the most selective (Carlsson, 1964).

Pheniprazine, which is structurally closely related to amphetamine, has also some slight amphetamine-like actions (Horita, 1959). Amphetamine increases the length of the activity in the canary studied with the present method (Wahlström, 1960). It is thus very possible that the initial increase in activity obtained with pheniprazine given late in the activity and during the rest period is an amphetamine-like effect. The direct central nervous stimulant effects of pheniprazine are weak compared with amphetamine (Horita, 1959) and this could explain the difference obtained between the high dose (100—175 mg/kg) and the low dose (60—85 mg/kg) of pheniprazine (Fig. 14 and 15).

The inhibition of the MAO accomplished with pheniprazine is irreversible. The inhibition of MAO in the liver of rats after a single dose of pheniprazine (20 mg/kg) was still evident after 8 days (80 % of normal activity) and the potentiating effect of 5-hydroxytryptophane on the pyrogenic response after pheniprazine in rabbits was still evident after nine days (Horita, 1959). In the rat and the rabbit pheniprazine thus has a long duration of action and the long duration of the effects seen in the canaries could be due to inhibition of the MAO.

Pargyline, although not a hydrazine, is an irreversible inhibitor of MAO with a long duration of action (Taylor et al., 1960). It seems as if in this regard it is similar to but more specific than the hydrazine inhibitors (Everett, Wiegand and Rinaldi, 1963, and following discussion). In the experiments performed in the canary it is hard to tell for how long a time the effects were present, due to a very large variation between the different experiments (measured as the standard error of the mean). The effects obtained with pargyline on the activity and rest were thus very variable but they seemed to have lasted no longer than with pheniprazine (approximately 14 days).

Pargyline in large doses initially gives a reduced motor activity in many species (Everett, Wiegand and Rinaldi, 1963). This clearly was

the case in the canary too after 350 mg/kg orally. What is remarkable is that the birds showed this decrease for at least 48 hours. The birds were fully capable of using the perches but only in 1 out of 11 experiments did they choose darkness. The question whether they were awake or not when sitting on the day perch cannot be answered with any degree of certainty. The birds did not use the normal sleeping position with the bill under a wing but canaries can sleep in other positions (Eckstein 1940). The birds probably slept during at least the main part of the long times spent on the day perch. This assumption is strengthened by the few experiments where the birds did choose between light and darkness and the rest period (darkness) was increased. This increase of the rest period was also evident when the regular circadian rhythm had reappeared.

Nialamide is also an irreversible MAO inhibitor of the hydrazine type. In mice the effect, measured as the protection against reserpine facilitation of metrazol convulsions, lasts for more than 11 days (Rowe et al. 1959). In the few experiments performed in the canary the pre-experimental relationship between activity and rest had not returned after 15 circadian periods. The effects of the length of the circadian period are hard to evaluate as the single bird used very often spontaneously changed the length of the circadian period.

The general pattern of the effects obtained with MAO inhibitors on the self selected rhythm in the canary is an initial decrease in the activity (phase I), which is more marked if the drug is given early in the activity than if it is given late. There is also a secondary reversal with an increase in the activity and a corresponding decrease in rest which is more marked when the inhibitor is given late in the activity. This generalization is slightly modified for pargyline where phase II is absent after administration as an AM dose and only slightly pronounced after a PM dose. With regard to the circadian period there seems to be a slight increase immediately after the drug administration which in the following circadian period is compensated in such a manner that the net effect on the waking up times is on the average negligible.

The fact that the increase in activity, which is a late effect, is dependent on the state of the bird when the drug was given several days earlier is puzzling and merits further investigation.

The MAO inhibitors are generally described as causing an increase in locomotor activity, usually called excitation (see for instance Spector 1963, Plummer and Furness 1963). It must be pointed out that an

periods were lengthened and they did not return to the pre experimental value in those shown in the figure

4 Discussion

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the case in the canary too after 300 mg/kg orally What is remarkable is that the birds showed this decrease for at least 48 hours The birds were fully capable of using the perches but only in 4 out of 11 experiments did they choose darkness The question whether they were awake or not when sitting on the day perch cannot be answered with any degree of certainty The birds did not use the normal sleeping position with the bill under a wing but canaries can sleep in other positions (Eckstein 1940) The birds probably slept during at least the main part of the long times spent on the day perch This assumption is strengthened by the few experiments where the birds did choose between light and darkness and the rest period (darkness) was increased This increase of the rest period was also evident when the regular circadian rhythm had reappeared

Nialamide is also an irreversible MAO inhibitor of the hydrazine type In mice the effect measured as the protection against reserpine facilitation of metrazol convulsions lasts for more than 11 days (Rowe et al 1959) In the few experiments performed in the canary the pre experimental relationship between activity and rest had not returned after 15 circadian periods The effects of the length of the circadian period are hard to evaluate as the single bird used very often spontaneously changed the length of the circadian period

The general pattern of the effects obtained with MAO inhibitors on the self selected rhythm in the canary is an initial decrease in the activity (phase I) which is more marked if the drug is given early in the activity than if it is given late There is also a secondary reversal with an increase in the activity and a corresponding decrease in rest which is more marked when the inhibitor is given late in the activity This generalization is slightly modified for pargyline where phase II is absent after administration as an AM dose and only slightly pronounced after a PM dose With regard to the circadian period there seems to be a slight increase immediately after the drug administration which in the following circadian period is compensated in such a manner that the net effect on the waking up times is on the average negligible

The fact that the increase in activity which is a late effect is dependent on the state of the bird when the drug was given several days earlier is puzzling and merits further investigation

The MAO inhibitors are generally described as causing an increase in locomotor activity usually called excitation (see for instance Spector 1963 Plummer and Furness 1963) It must be pointed out that an

increase in the length of the activity in this investigation is not synonymous with increased locomotor activity. The intensity of the locomotor activity which was obtained from the hops on the day perch during the activity was not quantitatively measured and no values of it were obtained. After the initial decrease, which was clearly evident with pargyline and nialamide, the locomotor activity seemed essentially uninfluenced. However, intense activity on the perches is hard to quantify (Mewaldt, Morton and Brown, 1964).

There is no reason to doubt that the influence of the length of activity and rest in these experiments was connected to the MAO inhibiting property of the drugs used. All three of the drugs caused similar changes. It is interesting that reserpine also caused a profound change in activity and rest but had very little influence on the length of the circadian period (Wahlström, 1964 c). It thus seems as if activity and rest within the circadian period are influenced by monoaminergic mechanisms.

CHAPTER IV

EFFECTS OF BARBITURATES ON THE CIRCADIAN RHYTHM

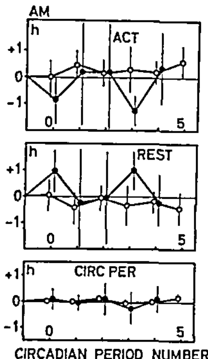
1 Results with pentobarbital

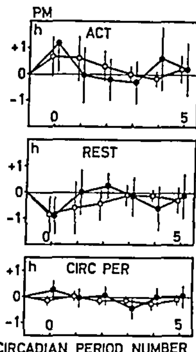
Pentobarbital has been given in two different doses Thirty two experiments with 20 mg/kg and 11 experiments with 25 mg/kg have been evaluated Thirteen birds have been used of which 2 were females Eleven experiments have been discarded due to technical reasons 2 as the birds died (after 20 mg/kg) and 9 as the birds had no circadian rhythm before or after the drug

After a dose of 25 mg/kg there was often inability to use the perches due to incoordination This effect was usually maximal approximately half an hour after the drug administration and had more or less disappeared after an hour After a dose of 20 mg/kg these effects were much less prominent In some cases slight difficulties in the use of the perches have been recorded for an hour after the drug

The results with pentobarbital given late in the activity period are shown in Fig 18 (PM) The changes have been calculated using the average of the 5 circadian periods prior to drug administration as baseline The curves show the average changes of the experiments performed The drug was given during circadian period no 0 After PM administration of pentobarbital there was an increase in the activity and a corresponding decrease in the rest in circadian period no 0 There were no changes in the length of the circadian period This retardation of roosting after pentobarbital was more pronounced after the higher dose which more often produced clear signs of drunkenness

The corresponding changes after pentobarbital administered early in the activity period are shown in Fig 18 (AM) 20 mg/kg had no effect at all but after 25 mg/kg there was an increase in the rest and a decrease in activity There was no change in the circadian period The birds did roost earlier after 25 mg/kg pentobarbital It is a little doubtful if this effect really was due to the drug as there was a similar change in activity and rest in circadian period no 3 Furthermore 20 mg/kg had no effect at all





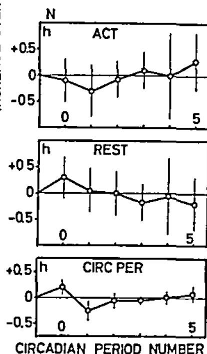
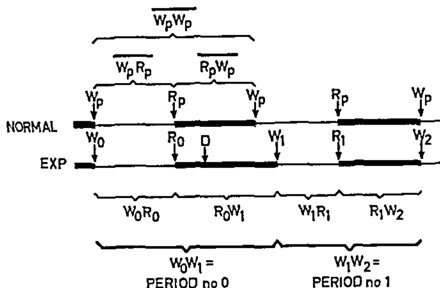


Fig 18 Effect of a single oral dose of pentobarbital on the circadian rhythm Increases have been calculated over the average (shown below) of the 5 periods immediately prior to the drug administration in period no 0 Experiments with 25 mg/kg marked ● and with 20 mg/kg O Vertical bars (sometimes plotted in only one direction) 2 × S E Adm time is the average time from waking up to drug administration

	AM O	AM ●	PM O	PM ●	N O
Act h	10 81	13.50	12.24	13 01	11 46
S E	0 82	2 49	1 27	1 93	0 37
Circ per h	23.32	24 31	23 83	24 15	23.81
S E	0 22	0 66	0 47	0.37	0 11
Adm time h	3 58	2 80	9 91	11 60	10 05
n	5	5	6	6	21[b]
Exp with 40 W	0	5	1	5	1

[a]/n= 4 from period no 2

[b]/n=19 from period no 3



Fi 19 Schematical illustration of the effects of barbiturates given during the rest period (N) on the circadian rhythm The symbols used in the pre experimental rhythm are identical with those in Fig 2 W_0=waking up starting period no 0 in which the drug was given R_0=roosting in period no 0 D=time at which the drug was given W_1=waking up starting period no 1 R_1=roosting in period no 1 Activity rest and circadian period are denoted W_0R_0 R_0W_1 and W_0W_1 in period no 0 and W_1R_1 R_1W_2 and W_1W in period no 1 The difference between these and the corresponding pre experimental values are seen in Fig 18 and 20

The results obtained with pentobarbital given during the rest period (N) are of special interest They are shown in Fig 18 (N) In Fig 19 these results are also schematically illustrated The letters in this figure will be used to denote the various changes The rest was increased in period no 0 ($R_0W_1 > \overline{R_pW_p}$) There was no change in activity the birds put out the light within 15 min after administration of the drug The waking up which started period no 1 (W_1) was delayed This is demonstrated by the slight but significant lengthening of circadian period no 0 ($W_0W_1 > \overline{W_pW_p}$) In circadian period no 1 the activity was decreased ($W_1R_1 < \overline{W_pR_p}$) by nearly the same amount as the rest was increased in period no 0 This means that the roosting in period no 1 (R_1) appeared at the time expected from the pre experimental period The length of the circadian period no 1 (W_1W_2) decreased with the same amount as it was increased in period no 0 ($W_0W_1 + W_1W = 2\overline{W_pW_p}$) This means that the waking up which started circadian period no 2 also appeared at the time expected from the pre experimental pe

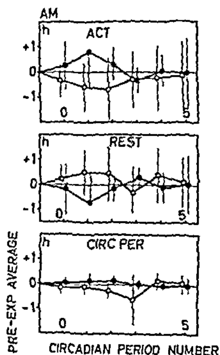
riods The only thing which thus happened to the rhythm was a delay in the waking up starting circadian period no 1 (W_1), and this delay did not influence the following waking up times at all They appeared at the clock time expected from the pre experimental periods If the barbiturate induced delay had been an EPD instead (c f Fig 2) the end of it would have been taken as a new starting point for the clock controlling the waking up times and circadian period no 1 would have been of pre experimental length

The delay in the waking up depended on neither the time during the rest period when the drug was given nor on the pre experimental length of the circadian period

2 Results with barbital

Barbital which is more long acting in most species has also been given in two different doses In 12 experiments in two birds (no 38 and 39) there was a very marked increase in rest (rest longer than 19 hours) after the drug and these experiments are shown separately The remaining experiments with shorter rest periods consisted of 29 with 100 mg/kg (including 2 experiments in bird no 38 and 9 experiments in bird no 39) and 30 with 75 mg/kg (including 3 experiments in bird no 38 and 4 experiments in bird no 39) A total of 9 birds were used of which one was an established female Six experiments were discarded for technical reasons 4 other experiments because the birds did not use the night perch after the drug but sat still on the day perch during the time they normally rested and a further 4 experiments since the birds did not show an overt rhythm before or after the drug Six more experiments were discarded in the series where the drug was given during the rest period (N) The reason for this was that some of the N experiments were performed late in the rest period If after such an experiment there was light in the cage after the drug administration or if the bird left the night perch within an hour after it (the drug was given at least two hours before the calculated waking up) the experiment could not be used since the following waking up seemed to be influenced by this exposure to light late in the rest period This is the mirror image of the EPD phenomenon and is under investigation It also happened if only water was given late in the rest period

The motor effects of barbital were similar to pentobarbital After a dose of 100 mg kg there was often incoordination and the birds were



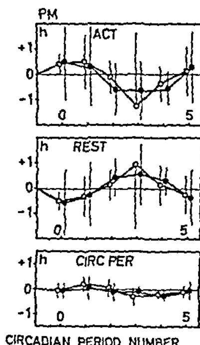


Fig. 20. Effect of a single oral dose of barbital on the circadian rhythm. Increases have been calculated over the average (shown below) of the 5 periods immediately prior to the drug administration in period no. 0. Experiments with 100 mg/kg marked ● and with 75 mg/kg ○. Vertical bars (sometimes plotted in only one direction) 2 × S.E. Adm. time is the average time from waking up to drug administration.

	AM ○	AM ●	PM ○	PM ●	N ○	N ●
Act. h	12.71	12.65	12.75	12.86	11.49	12.59
S.E.	0.81	0.54	1.06	0.62	0.38	0.53
Circ. per. h	23.97	23.88	23.93	23.95	23.91	23.94
S.E.	0.24	0.12	0.25	0.24	0.07	0.19
Adm. time h	2.56	2.59	10.24	9.59	19.44	16.51
n	8	11[b]	6	10	16	8[d]

[a]/n=7 in period no. 3 and then 6

[b]/n=10 from period no. 3

[c]/n=14 in period no. 2 and then 13

[d]/n=7 in period no. 3 and 4 and then 6

unable to use the perches These effects had usually disappeared after 1 to 2 hours After a dose of 75 mg/kg these signs of intoxication were much less pronounced and on only a few occasions were the birds unable to use the perches for one hour after the drug administration

Fig 20 (AM) shows the main results with barbital administered early in the activity The two dose levels *seemingly had opposite effects* on the length of the activity and rest none of them statistically significant As the dose levels were rather close it is unlikely that they should really give the opposite effects The circadian period was largely uninfluenced

When barbital was administered late in the activity (Fig 20 PM) the changes in activity and rest were slight but similar with the two doses of barbital used Activity was first increased and then decreased The increase in activity and decrease in rest in circadian period no 0 was of the same magnitude as the one obtained with pentobarbital The *circadian periods were mainly uninfluenced*

The changes obtained in the circadian rhythm when barbital was administered during the rest is shown in Fig 20 (N) In circadian period no 0 there was an increase in activity after a dose of 100 mg/kg This without doubt was due to the incoordination The birds *were in many cases unable to use the night perch after the drug administration* A dose of 75 mg/kg had no such effect Of much greater interest is the change in the circadian period As in the case of pentobarbital barbital caused a delay in the waking up after the drug administration shown as a lengthening of the circadian period ($W_0W_1 > \overline{W_pW_p}$ Fig 19) *The following waking up time was however* not changed compared with the preexperimental ones the next circadian period was correspondingly decreased ($W_0W_1 + W_1W_2 = 2\overline{W_pW_p}$ Fig 19) This decrease was due to a shorter activity the roosting in circadian period no 1 was not shifted compared with the preexperimental roosting *This effect was clearly dependent on the dose of barbital* Similar to the effects of pentobarbital the end of the barbital induced delay was not taken as a starting point for a circadian period of normal length which would have happened after a corresponding FPD (see chapter II)

In Fig 21 are shown the waking up times (starting hour of activity) in the experiments where there were long rest periods The waking up times are plotted for consecutive activity periods Barbital was administered during the circadian period which followed the waking up denoted circadian period no 0 Changes similar to those just des

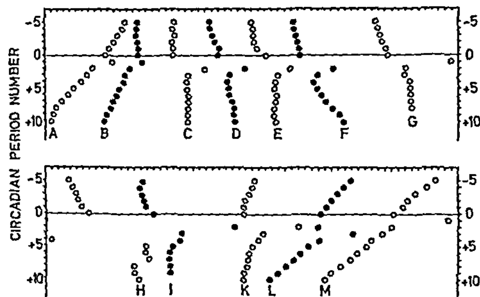


Fig 21 Effect of a single oral dose of barbital on waking up times in experiments with long rest periods The horizontal scale is divided into hours The drug was given during circadian period no 0 Experiment L and M were performed in bird no 38 the others in bird no 39 Experimental conditions

Experiment	A	B	C	D	E	F	G	H	I	K	L	M
Dose mg/kg	100	100	5	75	75	75	100	100	100	100	100	100
Administered	AM	AM	N	N	N	N	N	N	PM	PM	PM	PM
Time of waking up in circ per no —5	10^{54}	19^{54}	13^{4}	9^{44}	13^{04}	18^{2}	0^{43}	9^{45}	10^{8}	23^{4}	19^{34}	7^{30}

cribed for barbital administered during the rest are seen in experiments A B G and M The first waking up was retarded after the drug but afterwards the waking up times returned close to their expected clock time In the other experiments one or more activity periods had dis appeared The birds were sitting on the night perch for very long times In these experiments the first waking up after the long rest was usually retarded (experiments C D E F I and K) the only ex ception were experiments H and L After this first waking up there was usually a gradual return to the time expected from the pre experi mental waking up times (experiments C D E F I K and also to some degree in H) In several experiments they came back nearly exactly

to the position they should have had if there had been no disturbance of the rhythm (experiments D F H and K) In experiments L and I they nearly reached the same position but in experiments C and L they remained retarded by a couple of hours compared with the pre experimental waking up times

The reason why these birds sometimes reacted to barbital with a long rest period and sometimes not is not known It could be a seasonal change in the birds as long rest periods only occurred in experiments performed from late spring to early autumn However bird no 38 sometimes showed long rest periods and sometimes not in experiments run during the same months There was not an exceptionally large drug free interval before the experiments which gave these long rest periods

3 Discussion

The effects of barbiturates on sleep and wakefulness has recently been reviewed by Kleitman (1963 pp 293—304) In the human die meisten Schlafmittel in abends wirksamen Dosen führen tags nicht zum Schlafe (Renner 1923 p 259) The experiments of Hondelink (1932) in various species of finches also showed this If the birds were ready for sleep which was accomplished by keeping them awake with light it was much easier to induce sleep (as compared with anaesthesia) with the drugs he tested including a barbiturate The recording technique has been further developed by Dost (1934) and Pisker (1962) who both used this method to test hypnotic drugs in small birds and proposed it as a screening method

The present investigations showed that it was possible to prolong the sleep if the barbiturate was given during the rest period of the birds It was much harder to induce roosting at an earlier time than normal AM administration of pentobarbital gave a slight indication that this was possible with a high dose With barbital it was only possible on a few occasions in 2 birds (most marked in experiments A and B but also evident in experiments I K L and M Fig 21) Pentobarbital and barbital thus seldom induce roosting during the activity period in the canary but it is possible that these drugs can prevent external factors from delaying roosting at the normal time however this has not yet been tested

The delay in the waking up obtained with the barbiturates when they were administered during the rest is very interesting After doses

of 100 mg/kg of barbital the birds chose darkness for an average of 1 5 hours longer than they used to in the pre experimental rhythm If this darkness had been forced on the bird by external means (results with a corresponding EPD are seen in Table I chapter II) the birds would have started the clock controlling the waking up times approximately when the light returned and would have had a normal circadian period to the next waking up There would have been a permanent displacement of the starting points of the circadian periods After barbital in contrast the following circadian period was shortened and the waking up times returned to the clock time expected from the pre experimental periods there being no permanent displacement Similar but not so pronounced changes occurred in the other N experiments with barbiturates

When barbital induces a long rest period similar events occur The waking up times after the long rest period tend to appear at the expected time In this respect the results are rather similar to those obtained with reserpine (Wahlstrom 1964 c) There is however a difference after the loss of one or more whole activity periods the pre experimental length of the circadian period was immediately re established in 7 out of 13 experiments with reserpine After barbital the return to pre experimental length of the circadian period was gradual in 7 out of 8 experiments where one or more activity periods were lost In this connection it is interesting to note that barbital is extremely slowly excreted in at least one avian species namely the chicken (Koppanyi Murphy and Gray 1934)

CHAPTER V

SUMMARY AND CONCLUSIONS

The following summary is based upon the present (I) and two earlier papers Wahlström 1964 b (II) and Wahlström 1964 c (III)

The aim of these investigations in the canary has been to analyze the mechanisms controlling the self selected endogenous circadian rhythm of activity and rest Indications have been obtained that the waking up is controlled by a clock mechanism which is partially independent of the mechanism which controls the division of the circadian period into activity and rest

1 The normal rhythm (II)

No influence of small temperature variations handling in the preceding activity period and other external Zeitgebers on the *waking up* could be established *Roosting* however was influenced by handling late in the activity In the normal rhythm the length of the *circadian period* (from one waking up to the next) lies around 24 hours (21—26 5 h) Long time changes of the circadian period occur but usually it is remarkably constant Short time variability is rather small (coefficient of variation 0 2—4 %) and thus the waking up usually is very regular Long time changes of *activity* and *rest* are more pronounced Short time variability is also larger depending mainly on the irregularities in the roosting In birds with divided activity (rest periods interposed in the activity) an earlier roosting does not have a direct advancing influence on the following waking up There is thus no direct connection between roosting and waking up in the normal rhythm

2 Influence of drugs (I and III)

A single dose of reserpine (III) profoundly altered the relationship between activity and rest for 3—4 periods The amount of rest was

increased In several experiments one or more activity periods disappeared In spite of this the length of the circadian period was largely uninfluenced The clock time at which the birds woke up after reserpine was usually only slightly different from the expected one

Single doses of the monoamine oxidase inhibitors (I) (pheniprazine pargyline and nialamide) as a rule first decreased activity a few days and then increased it Opposite changes were obtained in the rest period. The effects lasted approximately a fortnight Surprisingly the increase in activity was more pronounced if the drug was given late (PM) compared with early (AM) in the activity The length of the circadian period was usually slightly longer immediately after the drug and then slightly shorter for some periods afterwards These changes in some of the individual experiments were rather striking (Wahlström 1964 a) The average net result was however that the waking up times returned to the clock time to be expected from the pre experimental periods There was thus no resetting of the clock mechanism controlling the waking up times and it seemed as if the mechanism was able to make up for time gained or lost after the drug administration

Results with pentobarbital and barbital are given in (I) Some experiments with barbital in 2 birds showed a substantial increase in the rest period Sometimes 1 or 2 activity periods disappeared The clock time to which the waking up times returned was not markedly different from the expected one Even in these experiments the clock controlling the waking up times was not reset

In the other experiments with barbital it was only possible to induce a longer rest period in experiments where barbital was given during the rest period (N) and in these the birds woke up later The following circadian period was however shortened with the same amount and there was not net result on the subsequent waking up times The clock controlling the waking up times was not reset Pentobarbital gave similar results When pentobarbital was given during the activity period the roostings appeared earlier in one series of experiments The results of this series are however not without objections

3 Influence of an enforced period of darkness (EPD) (I)

In contrast to the absence of a resetting effect of drugs the clock could be easily reset by an EPD The resetting effect occurred in the

The effects of drugs on short term timing behaviour can be assessed by a Skinner box technique (see for instance Sidman 1955 Dews 1955) The parmacological data from the self selected rhythm of the canary are however too limited at present to make a comparison worthwhile

Finally it must be mentioned that Aschoff and Wever (1962) have used self selection of light and darkness with human volunteers as subjects The rhythm obtained consisted of one activity period (some times divided) and one rest period Waking up was more regular than going to bed The circadian period in the few subjects tested so far was always longer than 24 hours A circadian period longer than 24 hours despite access to a watch was also obtained in one subject who lived in a cave for 105 days in a similar but less well controlled study (Mills 1964)

There are thus several similarities between the self selected rhythm in the human and the canary and these two species seem to have at least one more trait in common than the propensity to burst into song at irregular intervals

LIST OF ABBREVIATIONS

General abbreviations

Common abbreviations not mentioned here are in accordance with the rules of Acta Physiologica Scandinavica

Act.	Activity
Adm Time	Average time from waking up to drug administration
AM	Treatment early in the activity period
Av	Average
b	Regression coefficient
Circ per	Circadian period
EPD	Enforced period of darkness
Exp	Experiment al
MAO	Monoamine oxidase
N	Treatment in the rest period
P	Probability
PM	Treatment late in the activity period
	Correlation coefficient

Abbreviations used to denote special features in the circadian rhythm

All treatments were performed in circadian period no 0 The following periods were numbered consecutively

L	Time of first light in experiments with EPD s
R	Roosting
—	Index number refers to the circadian period after treatment in which the roosting occured
—	Index p refers to pre experimental roosting
W	Waking up
—	Index number refers to the circadian period after treatment which is started by the waking up
—	Index p refers to pre experimental waking up
—	Index a refers to attempted waking up
—	Index h refers to hypothetical waking up (one pre experimental circadian period before the following waking up)
DR	Delay of roosting index as for R
DW	Delay of waking up index as for W
LP	Time from first light to roosting index as for R.
LW	Time from first light to waking up index as for W

$\underline{RW}$	Rest period index as for R and W
$\overline{R_pW_p}$	Average of 5 pre experimental rest periods
W_aL	Length of EPD
$\underline{WR}$	Activity period index as for W and R
$\overline{W_pR_p}$	Average of 5 pre experimental rest periods
$\underline{WW}$	Circadian period index as for W
$\overline{W_pW_p}$	Average of 5 pre experimental circadian periods

In the experiments with EPDs (chapter II) the various series have been denoted D or L D and the *index of L denotes time of light prior to the EPD in sec* and *index of D length of EPD in hours*

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